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Serial No. 09/402,273

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In Re Application of:
Jorj Terry ULRICH et al.

Confirmation No. 5731

Serial No. 09/402,273

Group Art Unit 1644

Filing Date: December 13, 1999

Examiner Phuong N. HUYNH

Title: ALLERGEN FORMULATION

REPLY BRIEF

Karen Canaan, Esq.
Registration No. 42,382
REED & EBERLE LLP
800 Menlo Avenue, Suite 210
Menlo Park, California 94025
(650) 330-0900 Telephone
(650) 330-0980 Facsimile

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REPLY BRIEF

Pursuant to 35 U.S.C. § 134(a) and 37 C.F.R. § 1.193(b)(1), with this Reply Brief, applicants are replying to the Examiner's Answer, mailed from the Office on May 5, 2004. In the Examiner's Answer, the Examiner reversed the final rejection of the claims under 35 U.S.C. § 112, first paragraph; accordingly, the only rejections remaining for this application are the obviousness rejections 35 U.S.C. § 103(a) set forth in the final Office Action of February 26, 2003, and repeated in the Examiner's Answer. This Reply Brief is timely filed on July 5, 2004, a date two months from the May 5, 2004, mailing date of the Examiner's Answer. Pursuant to 37 C.F.R. § 1.192(c)(9), the claims on appeal are attached at Appendix A.

REAL PARTY IN INTEREST:

The real party in interest for this brief is Allergy Therapeutics Limited, by way of an assignment from the inventors to Allergy Therapeutics Limited, recorded with the Office at Reel 010448, Frame 0312, on December 13, 1999.

RELATED APPEALS AND INTERFERENCES:

There are no related appeals and interferences for this matter.

STATUS OF CLAIMS:

Claims 1, 2, 6-8 and 15-23 are pending and are appealed herein. The claims stand finally rejected as follows:

1. claims 1-2, 6-8, 15-17, and 19-23 under 35 U.S.C. § 103(a) as obvious over WO 96/34626 in view of WO 92/16556 and U.S. Patent No. 5,795,862;
2. claim 18 under 35 U.S.C. § 103(a) as obvious over WO 96/34626 in view of WO 92/16556 and U.S. Patent No. 5,795,862 as applied to claims 1-2, 6-8, 15-17, and 19-23 and further in view of Marsh, WO 92/16556, U.S. Patent No. 5,750,110, and Hoyne et al.;
3. and claims 1 and 23 under 35 U.S.C. § 103(a) as obvious over WO 96/34626 in view of Holen et al., WO 92/16556, U.S. Patent No. 5,750,110, and Hoyne et al.

STATUS OF AMENDMENTS:

No claim amendments were submitted after final rejection.

SUMMARY OF THE INVENTION:

The present invention was first described in PCT application WO 98/44947, filed on April 3, 1998. This PCT application claimed international priority back to April 5, 1997, based upon GB 9706957.9, and was published on October 15, 1998. At the time the invention was made, the inventors, Jorj Terry Ulrich and Alan Worland Wheeler, were under an obligation to SmithKline Beecham and consequently, the priority document, WO 98/44947, was assigned to SmithKline Beecham. The instant U.S. patent application was filed on December 19, 1999. At the time the instant U.S. application was filed, the inventors were no longer under an obligation to SmithKline Beecham and consequently, the inventors assigned their interest in the U.S. application to Allergy Therapeutics Ltd., the latter having acquired SmithKline Beecham's rights in the invention that is the subject matter of this application on or about June 1998.

In general, the present invention relates to novel formulations for use in desensitization therapy of allergy suffers (p.1, ll. 3-4). Desensitization therapy results in a changed immunological response specific for a particular allergen such that the symptoms of the allergy from the allergen are ameliorated (p.1, ll. 5-7). Traditionally, the changed immunological response was associated with an increase in allergen specific antibodies; however, the present invention is premised on the theory that the more important immunological change in an allergic response concerns allergen specific T lymphocytes (p.1, ll. 6-11). Specifically, of the two subclasses of T lymphocytes, Th_1 and Th_2, it is believed that Th_2 activity is

increased in an allergic subject and that this increase leads to an increase in two important components of the allergic syndrome: high allergen specific IgE antibody level and greater eosinophil activity (p.1, ll. 12-16). The present invention is premised on the theory that when there is greater allergen specific Th_1 over Th_2 activity, immunotherapy of an allergic patient is improved (p.1, ll. 17-19). One substance that can enhance Th_1 over Th_2 activity in the blood of an allergic patient is 3-de-O-acylated monophosphoryl lipid A ("3-DMPL") (p.1, ll. 26-31). As seen in the table at page 6, the combination of allergen (i.e., ovalbumem) in combination with tyrosine and MPL (row two of the table) induces less allergen specific IgE antibodies than combinations of allergen and tyrosine (row one of the table) or allergen and MPL (row three of the table) indicating a better ratio of Th_1 cell induction over Th_2 cell induction (where IgG_{2a} and IgG_{2b} are indicative of a Th_1 response and IgG_1 and IgE are indicative of a Th_2response).

Amongst this background, the claimed invention relates to a pharmaceutical composition capable of selectively enhancing a Th_1 response over a Th_2 response, comprising tyrosine, an allergen or allergen extract, and 3-DMPL (p.1, ll. 32-33; claim 1). Within the context of the claimed invention, the allergen or allergen extract is coated with and/or adsorbed into tyrosine (p.1, ll. 33-34; claims 2 and 6-8).

The allergen of the claimed invention may be optionally modified through reaction with a cross-linking agent such as a dialdehyde or more particularly, a glutaraldehyde (p.2, ll.15-16; claims 15-18). When a cross-linking agent is used, the allergen is typically modified by treatment with the cross-linking agent in an aqueous solution at a pH of between 5 and 10 and temperatures between 0°C and 100°C for up to ten hours (p.2, ll. 24-28). In one embodiment, the allergen may be modified at a pH of 7 and a temperature between 4°C and 37°C (such as room temperature) for approximately two hours (spec., p.2, ll. 26-28). If glutaraldehyde is used as the cross-linking agent, it will typically be in the range of 50:1 to 2:1, with a preferred range of 10:1 (p.2, ll. 28-29).

To adsorb the allergen, the claimed invention uses tyrosine. The tyrosine is added to the allergen by mixing a solution of modified or unmodified allergen with a solution of tyrosine in a strong aqueous solution, such as hydrochloride or another inorganic acid (p.2, ll. 31-34; p.3, l.16). The solution of allergen typically contains between 0.1 μg/mL and 1000 μg/mL allergen protein with the ratio of allergen to tyrosine in the range of approximately $1:4 \times 10^5$ to $1:1 \times 10^2$ w/w (p.2, ll. 34-37). In conjunction with adding the acidic tyrosine solution to the allergen, the mixture of the allergen, the mixture is neutralized with an appropriate base and if necessary, a buffering agent (p.3, ll. 1-7). Neutralization is typically between pH 4.0 to no higher than pH 7.5, with a pH of 6.5 to 7.5 being most common for many allergens (p.3, ll. 2 and 12-14). The result of the neutralization is the immediate precipitation of the tyrosine and the occlusion or adsorption of the allergen (p.3, ll. 15-16). The resulting precipitate is removed from the solution by centrifugation or filtration and then washed with a solution of, for example, phenol-saline

before being resuspended in a physiologically acceptable carrier such as phenol-saline or water to produce an injectable composition suitable for use in desensitization therapy in combination with 3-DMPL (p.3, ll. 19-22).

The allergen of the claimed invention may be derived from any allergy causing substance or source, alone or in combination; such substances or sources include, for example, pollen (e.g., ragweed or birch pollen), food, insect venom, mold, animal fur, or house dust mite (*D. farinae* or *D. pteronyssinus*) (p.2, ll. 9-12; claims 19-23).

<u>THE CITED REFERENCES:</u>

1. WO 96/34626

WO 96/34626 teaches a pharmaceutical composition that includes tyrosine and a modified allergen or allergen extract such as glutaraldehyde treated (polymerized) ragweed, birch pollen, food, mold, or house dust mite derived from *D. farinae* or *D. pteronyssinus*. This reference does ***not*** teach or suggest including 3-DMPL or any other adjuvant in the disclosed pharmaceutical composition and also does ***not*** teach or suggest that an enhancement of a Th_1 response over a Th_2 response may be effective in increasing the effects of desensitization therapy for allergy sufferers.

2. WO 92/16556

WO 92/16556 teaches an HIV/AIDS vaccine formulation consisting of a viral antigen (a single glycoprotein of 160 Kd - "gp160") with the addition of 3-DMPL. The use of the 3-DMPL in this reference is disclosed as an adjuvant to "present immunogens effectively to the host immune system such that both arms of the immune response (neutralising antibody and effector cell mediated immunity (DHT)) are produced" (p.8, ll. 22-26). This reference does ***not*** teach or suggest that the 3-DMPL is used to enhance a Th_1 response over a Th_2 response. Further, as is self-evident from the disclosure, this reference teaches antiviral immunotherapy ***not*** allergy immunotherapy.

3. U.S. PATENT NO. 5,795,862 (THE '862 PATENT)

U.S. Patent No. 5,795,862 teaches a formulation and method for isolating ectoparasite saliva proteins and a composition for detecting allergic dermatitis in an animal (col. 1, ll. 15-17). At col. 42, l.32, the composition of the '862 Patent is disclosed as including "Ribi adjuvant," which may serve as a carrier to enhance the immune response of an animal to a specific antigen (col. 42, ll. 19-25). Not only is there ***no*** indication in this reference that the "Ribi adjuvant" is 3-DMPL, there is also ***no*** disclosure in this reference on how or in what way the adjuvant increases the immune response of the animal.

4. MARSH

"Preparation and Properties of 'Allergoids' Derived from Native Pollen Allergens by Mild Formalin Treatment" ***Int. Arch. Allergy*** **41:199-215 (1971)**

Marsh is a paper that describes the production and properties of a new type of allergen derivative, termed an allergoid (p.199, ¶ 4 to p.200). For allergens, Marsh's system uses highly purified rye grass pollen antigen (p.200, ¶ 1). The allergoid of Marsh is prepared by incubating the allergen with dilute formaldehyde in the presence or absence of additives that become incorporated chemically into the resultant derivatives (p.201, ¶ 1). Without additives, methylene bridge linkages of varying stabilities are formed between amino and reactive aromatic residues on the allergen (such as amido or guanidine). With additives, the reactions involve cross-linkage between additive and allergen rather than intra-protein cross-linkage (p.200, ¶ 2). Due to the functional groups affected by formaldehyde modification, Marsh provides that the formalinized allergens, i.e., the allegoids, are considerably more acidic than native allergen (p.202, ¶ 1).

Marsh studied the residual allergenicities of two formalinized (32-day) derivatives of grass pollen (the normal and lysine allergoids) relative to native allergen by direct intradermal skin tests on grass pollen-allergic individuals (p.202, ¶ 4; Table 1). From this data, Marsh concludes that the two derivatives appeared to possess 0.01% to 0.5% of the allergenic activity of the native allergen (p.202, ¶ 4).

5. U.S. PATENT NO. 5,750,110 (THE '110 PATENT)

The '110 Patent teaches a vaccine formulation that includes 3-DMPL and QS21 (a saponin derivative) for the treatment of viral diseases, such as immunodeficiency viruses, herpes viruses, cytomegalovirus, varicella zoster virus, hepatitis virus, respiratory sunctial virus, human papilloma virus, influenza virus; bacterial disesases, such as salmonella, neisseria, borellia, Chlamydia, bordetella; or parasitic diseases, such as plasmodium or toxoplasma (col. 1, ll. 56-57). The '110 Patent discloses that the combination of 3-DMPL and QS21 *synergistically* enhance immune responses to a given antigen (col. 1, ll. 20-22). The '110 Patent discloses such immune responses as induction of cytotoxis T cells ("CTLs") in response to the malarial antigen RTS.S (col. 1, ll. 23-17) and gamma interferon ("IFN-γ") in response to the herpes simplex antigen gD_2t (col. 2, ll. 33-35). The '110 Patent mentions that Th_1 responses are induced via IFN-γ (col. 2, ll. 40-43). The '110 Patent discloses that the synergism between the 3-DMPL and QS21 in the vaccines is two-fold greater than the effect of the individual adjuvants in the vaccines (table and text at col. 5, ll. 19-37).

6. HOYNE ET AL.

"Peptide-Mediated Regulation of the Allergic Immune Response," ***Immunology and Cell Biology*** **74:180-186 (1996)**

Hoyne et al. is a review paper that examines *inter alia,* the possibility of reprogramming immune responses by promoting Th_1 responses *instead* of Th_2 responses (Abstract). Hoyne et al. arises at this postulate by observing that the desensitized allergic patients display a decrease in Th_2 cytokine production (p.180, col. 1, ¶ 2 and col. 2, bottom of the page, and p.183, col. 1, ¶ 2). Hoyne et al. suggests that clinical desensitization may involve replacing IL-4 secreting cells with specific CD4+ T cells that secrete IFN-γ, which would help down-regulate ongoing Th_2 responses *in vivo* (p.183, col. 2, top of page). Based on this observation, Hoyne et al. postulates that by decreasing the functional response of Th_2/Th_0 cells, improved clinical symptoms for allergy patients will result. Hoyne et al. suggests that this may be accomplished through the mucosal delivery of antigens to silence existing memory Th_2/Th_0 cells and then reprogramming the immune response towards a Th_1 phenotype by co-adminstering allergen in the presence of IL-12 or IFN-γ or by immunizing with recombinant live vaccine vectors such as mycobacteria expressing defined allergens or fragments (p.183, col. 2, ¶ 21). Hoyne et al. conclude that the development of peripheral tolerance memory Th_2 cells *in vivo* has been controversial and little is known about the plasticity of the human peripheral repertoire following desensitization. Noting that it is much easier to manipulate the effector function of naïve cells rather than memory T cells and that allergic sensitization frequently occurs during infancy or early childhood, Hoyne et al. suggest that it may be beneficial to target naïve cells by vaccinating children who are at high risk of developing an allergic disease due to family history of allergy. Hoyne et al. postulate that vaccination could either induce peripheral tolerance or induce allergen-specific Th_1-type responses (p.184, col. 1, ¶ 2).

7. HOLEN ET AL.

"Specific T Cell Lines for Ovalbumin, Ovomucoid, Lysozyme and Two OA Specific Epitopes, Generated from Egg Allergic Patients' PBMC" ***Clin. Exp. Allergy*** **26(9):1080-1088 (1996) (Abstract)**

Holen et al. describes a study wherein peripheral blood mononuclear cells from hen egg allergic patients were investigated to determine the T cell epitopes responsible for the allergic response in the patients. Using various allergens of hen egg white for stimulation, long term cultures of enriched CD4+/CD8+ T cells (CD2+ > 95%) were prepared following primary proliferation responses. The long term cultures were observed for specificity, phenotype, cytokine profile, and IgE production. The allergen specific T cell lines were mapped using a panel of 13 synthetic peptides of ovalbumin. The study found that human T cells recognize ovomucoid, lysozyme, and ovalbumin epitope 105-122.

ISSUES ON APPEAL:

In the Appeal Brief, it was argued that the WO 96/34626 reference was disqualified as prior art and consequently, arguments were presented that did not include the WO 96/34626 reference. In this Reply Brief, applicants are explaining in further detail why the WO 96/34626 reference should be disqualified as prior art, but are also presenting arguments in response to the Examiner's obviousness rejections that consider the WO 96/34626 reference; accordingly, the following issues are to be considered on appeal:

I. whether the primary reference, WO 96/34626, is disqualified as prior art under 35 U.S.C. § 103(a); and

II. whether the following obviousness rejections under 35 U.S.C. § 103(a) represent a *prima facie* case of obviousness:

   A. claims 1-2, 6-8, 15-17, and 19-23 over WO 96/34626 in view of WO 92/16556 and U.S. Patent No. 5,795,862,

   B. claim 18 over WO 96/34626 in view of WO 92/16556 and U.S. Patent No. 5,795,862 as applied to claims 1-2, 6-8, 15-17, and 19-23 and further in view of Marsh, U.S. Patent No. 5,750,110, and Hoyne et al., and

   C. claims 1 and 23 over WO 96/34626 in view of Holen et al., WO 92/16556, U.S. Patent No. 5,750,110, and Hoyne et al.

GROUPING OF CLAIMS:

All claims stand and fall together for purposes of this appeal.

ARGUMENT:

I. THE DISQUALIFICATION OF THE WO 96/34626 REFERENCE AS PRIOR ART

The Examiner rejected applicants' arguments to disqualify the WO 96/34626 reference on the grounds that the WO 96/34626 reference is statutorily barred from being disqualified as a reference because it was published more than one year before the April 3, 1998, filing date of the PCT priority document, which the Examiner asserts is the effective date of the instant application (Ex. Answ., p.16, 2nd full para.). Taking this position, the Examiner did not find the Declaration of Alan Worland Wheeler to be persuasive and repeated the obviousness rejections from the final Office Action that include the WO 96/34626 reference.

Applicants respectfully disagree with the Examiner and will demonstrate herein why the priority date of the instant application is the April 5, 1997, filing date of the British priority document, rather than the April 3, 1998, filing date of the PCT application. With this showing, it will follow that the WO 96/34626 reference is not statutorily barred from being back-dated by the Declaration of Alan Worland Wheeler.

35 U.S.C. § 365(c) provides as follows:

> In accordance with the conditions and requirements of section 120 of this title, an international application designating the United States shall be entitled to the benefit of the filing date of a prior national application or a prior international application designating the United States, and a national application shall be entitled to the benefit of the filing date of a prior international application designating the United States.

The first sentence of this application clearly states that an international application designating the United States shall be entitled to claim benefit of the filing date of the national application from which it is based. In the instant case, the PCT application that resulted in this United States application was filed with a priority claim to the April 5, 1997, filing date of the British national phase application identified by GB 9706957.9. This priority claim should be evident in the official file. Accordingly, the effective date of the instant application is ***not*** the April 3, 1998, international filing date as asserted by the Examiner; rather, it is the April 5, 1997, priority date of the earliest filed national phase application. Because the November 7, 1996, international publication date of the WO 96/34626 reference is not more than one year prior to the April 5, 1997, priority date of the instant application, applicants submit that the WO 96/34626 reference can be disqualified as prior art against the claimed invention. For this reason, applicants respectfully request that this Honorable Board give full consideration to the Declaration of Alan Worland Wheeler, which presents evidence that the present invention predates the November 7, 1996, filing date of the WO 96/34626 reference and the arguments set forth in the Appeal Brief that addresses the Examiner's obviousness rejections sans the WO 96/34626 reference.

For purposes of completion, applicants take this opportunity to notify the Board that diligent efforts were made both prior to filing the Appeal Brief and prior to filing this Reply Brief to locate co-inventor Jorg Terry Ulrich and to submit a Declaration by co-inventor Ulrich in support of the prior invention of the subject matter of this application relative to the November 7, 1996, international publication date of the WO 96/34626 reference. The attached Declaration of Karen Canaan outlines the

diligent efforts that were made to contact Dr. Ulrich and how these diligent efforts failed to produce co-inventor Jorg Terry Ulrich (attached at Appendix B).

With respect to applicants attempts to disqualify the WO 96/34626 reference on the grounds of common ownership, applicants acknowledge that the Statement of Common Ownership submitted in the Appeal Brief requires verification that the WO 96/34626 reference was filed as a national stage application under 35 U.S.C. § 371(c). Because applicants have still not been able to verify that the WO 96/34626 reference was filed as a national phase application for the reasons set forth in the Appeal Brief, applicants again request that that this Honorable Board review the record to affirm that the WO 96/34626 reference was filed as a U.S. national phase application. If the WO 96/34626 reference was in fact filed as a U.S. national phase application, then applicants reiterate that the WO 96/34626 reference should also be disqualified as prior art on the grounds that the subject mater of the WO 96/34626 reference and the subject matter of the claimed invention were commonly owned at the time of the present invention was made.

Should this Honorable Board find that the WO 96/34626 reference is not disqualified as prior art, then the following arguments will show that even with the inclusion of the WO 96/34626 reference, the Examiner has still failed to establish a *prima facie* case of obviousness over the claimed invention.

II. THE NON-OBVIOUSNESS OF THE CLAIMED INVENTION OVER THE CITED ART

A. THE LEGAL STANDARD FOR OBVIOUSNESS

The *prima facie* case is a procedural tool which, as used in patent examination, means not only that the evidence of the prior art would reasonably allow the conclusion the Examiner seeks, but also that the prior art compels such a conclusion if the applicant produces no evidence or argument to rebut it. *In re Spada,* 911 F.2d 705, 15 USPQ2d 1655 (Fed. Cir. 1990). If examination at the initial stage does not produce a *prima facie* case of unpatentability, then without more, the applicant is entitled to a grant of the patent. *In re Oetiker,* 977 F.2d 1443, 24 USPQ2d 1443 (Fed. Cir. 1992).

A *prima facie* case of obviousness under 35 U.S.C. § 103 requires a showing that the cited prior art reference teaches or suggests the claimed combination and that the ordinary artisan would have a reasonable expectation of success at arriving at the claimed combination based *solely* on the teachings of the cited prior art reference. *In re Vaeck,* 947 F.2d 488 (Fed. Cir. 1991).

A prerequisite to making a finding on section 103 obviousness is determining what is prior art, in order to consider whether the differences between the subject matter sought to be patented and the prior art such that the subject matter as a whole would have been obvious at the time of the invention to one of ordinary skill in the art. *In re Clay*, 966 F.2d 656, 23 USPQ2d 1058 (Fed. Cir. 1992). If a cited reference

is not analogous art, it has no bearing on the obviousness of the patent claim. *Jurgens v. McKasy*, 927 F.2d 1552, 18 USPQ2d 1031 (Fed. Cir. 1991), *cert. denied*, 502 U.S. 902 (1991). Under the two step test for determining whether a prior art reference is nonanalogous and thus not relevant in determining obviousness, it must be determined: (i) whether the reference is "within the field of the inventor's endeavor"; and (ii) if not, whether the reference is "reasonably pertinent to the particular problem with which the inventor was involved." *In re Deminski*, 796 F.2d 436, 230 USPQ 313 (Fed. Cir. 1986). The claimed invention and the reference patents are within the same field of endeavor if they have essentially the same function and structure. *Id.*

It is well-established that even if a reference teaches every element of the claimed invention, the reference will not render the claimed invention obvious if the inventor *would not have been motivated* to turn to the teachings in the reference. *In re Rouffet,* 149 F.3d 1350, 1357, 47 USPQ2d 1453 (Fed. Cir. 1998). Although the Commissioner suggests that [the structure in the primary prior art reference] could readily be modified to form the [claimed] structure, '[t]he mere fact that the prior art could be so modified would not have made the modification obvious unless the prior art suggested the desirability of the modification. *In re Laskowski,* 871 F.2d 115, 10 USPQ2d 1397 (Fed. Cir. 1989).

It is also well-established that an obviousness analysis that relies upon the applicant's disclosure rather than the prior art reference is improper as being based upon an impermissible hindsight reconstruction. *In re Deuel,* 51 F.3d 1551, 34 USPQ2d 1210 (Fed. Cir. 1995). Similarly, the combination of elements from nonanalogous sources, in a manner that reconstructs the applicant's invention only with the benefit of hindsight, is insufficient to present a *prima facie* case of obviousness. *In re Oetiker, supra.*

Where an Examiner chooses to take notice of facts beyond the record for the *prima facie* case, those facts must be "capable of such instant and unquestionable demonstration as to defy dispute." *In re Alhert*, 24 F.2d 1099, 1091 (CCPA 1970). It is ***not*** appropriate for an Examiner to take official notice of facts without citing a prior art reference where the facts asserted to be well-known are not capable of *instant and unquestionable demonstration as being well-known. Id.* For example, assertions of technical facts in esoteric technology or specific knowledge of the prior art must always be supported by citation to some reference work recognized as standard in the pertinent art. *Id.*; *see also*, MPEP § 2144.03, 8th ed., Aug. 2001, Rev. Feb. 2003, pp. 2100-131 to 2100-132; *In re Grose and Flanigan*, 592 F.2d 1161, 1167-1168, 201 USPQ 57 (CCPA 1979) ("[W]hen the PTO seeks to rely upon a chemical theory, in establishing a prima facie case of obviousness, it must provide evidentiary support for the existence and meaning of the theory."). More importantly, it has also been established that an Examiner is not at liberty to assert that the state of the art is common knowledge; the state of the art must ***always*** be shown by way of documentary evidence. *See*, MPEP § 2144.03, p.2100-132; *In re Eynde, Pollet, and DeCat*, 480 F.2d

1364, 1370, 178 USPQ 470 (CCPA 1973) ("[W]e reject the notion that judicial or administrative notice may be taken of the state of the art. The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are not amenable to the taking of such notice."). All of these well-established principles of administrative notice were reiterated by the Federal Circuit in the important case, *In re Zurko*, 258 F.3d 1379, 1385, 59 USPQ2d 1693 (Fed. Cir. 2001) ("[T]he Board cannot simply reach conclusions based on its own understanding or experience – or on its assessment of what would be basic knowledge or common sense.").

B. CLAIMS 1, 2, 6-8, 15-17, AND 19-23 ARE NOT OBVIOUS OVER WO 96/34626 IN VIEW OF WO 92/16556 AND THE '862 PATENT

Of the claims identified in this rejection, claim 1 is the only independent claim, it reads as follows:

> A pharmaceutical composition capable of selectively enhancing a Th_1 response over a Th_2 response, comprising tyrosine, an allergen or allergen extract, and 3-DMPL.

Because all claims stand and fall together for purposes of this appeal, applicants will limit this discussion to the features of claim 1. The following discussion will demonstrate that claim 1 is not obvious over the cited reference; accordingly, with this showing, it will follow that all of the claims dependent on claim 1 are also not obvious over the cited references.

The WO 96/34626 reference teaches a pharmaceutical composition that includes tyrosine and a modified allergen or allergen extract such as glutaraldehyde treated (polymerized) ragweed, birch pollen, food, mold, or house dust mite derived from *D. farinae* or *D. pteronyssinus* for use in desensitization therapy of allergy sufferers. This reference does ***not*** teach or suggest including 3-DMPL or any other adjuvant in the disclosed pharmaceutical composition and more importantly does ***not*** teach or suggest that an enhancement of a Th_1 response over a Th_2 response may be effective in increasing the effects of desensitization therapy for allergy sufferers. Because of these deficiencies in the teachings of WO 96/34626, it follows that this reference alone does not render the claimed invention obvious.

The WO 92/16556 reference and the '862 Patent do not correct the deficiencies of WO 96/34626 for the reasons that follow.

The WO 92/16556 reference teaches an HIV/AIDS vaccine formulation consisting of the viral antigen gp160 with the addition of 3-DMPL. The use of the 3-DMPL in this reference is disclosed as an adjuvant to "present immunogens effectively to the host immune system such that both arms of the immune response (neutralising (sic) antibody and effector cell mediated immunity (DTH)) are produced"

(p.8, ll. 22-26). Contrary to the Examiner's assertion, this reference does ***not*** teach or suggest that 3-DMPL is effective to enhance a Th_1 response over a Th_2 response. Indeed, nowhere in WO 92/16556 is a Th_1 or a Th_2 response mentioned.

In the Examiner's Answer, the Examiner attempts to justify the relevance of this reference by asserting that the DTH referenced in WO 92/16556 is a Th_1 response (Ex. Answ., p.5, 1st full para., 1st sent.) and that the enhancement of a Th_1 response over a Th_2 response is an inherent functional property of 3-DMPL (Ex. Answ., p.5, 1st full para., last sent.).

With respect to the former, applicants have reviewed the section identified by the Examiner as well as the entire document in detail and have not found one incident in WO 92/16556 where DTH is expressly stated to be a Th_1 response. At page 29, line 10, WO 92/16556 states that formulations containing 3-DMPL in an oil/water ("o/w") emulsion are able to induce *a specific T cell response* as seen in Table 6; however, the specific T cell response is ***not*** identified as a Th_1 response in either the text or in Table 6. Table 6 shows the anti-HSV antibody response, as determined by ELISA titer and microneutralization titer, in chimpanzees vaccinated with 100 μg recombinant gp160 ("rgp160") and 3-DMPL o/w. The results of this table certainly do not teach or suggest that the 3-DMPL induces a Th_1 response; it merely shows that the vaccine, which includes 3-DMPL, produces an antibody response in the vaccinated chimps.

In light of the foregoing analysis, applicants can only conclude that the Examiner's statement that DTH is synonymous with a Th_1 response is a statement of common knowledge in the art. Because the Examiner's statement is not supported by any evidence in the official record of this case, applicants respectfully request that this Honorable Board disregard the Examiner's statement. *See, e.g.*, *In re Alhert*; *In re Grose et al.*; *In re Eynde et al.*; and *In re Zurko, supra*. Notwithstanding the foregoing request, applicants note that not only is it ***not*** common knowledge that DTH is synonymous with a Th_1 response, but literature that applicant is aware of shows that the Examiner's statement of common knowledge is in fact *not* an accurate statement. *See, e.g.*, Akahaira-Azuma et al., *Early Delayed-Type Hypersensitivity Eosinophil Infiltrates Depend on T Helper 2 Cytokines and Interferon-γ via CXCR3 Chemokines*, IMMUNOLOGY 111(3):306-317 (2004) (both Th_1 and Th_2 may be implicated in early delayed type hypersensitivity) (attached at Appendix C).

With respect to the latter, the Examiner's statement that the enhancement of a Th_1 response over a Th_2 response is a functional property of 3-DMPL is also a clear assertion of common knowledge in the art. Because ***nowhere*** in WO 92/16556 is it stated that the enhancement of a Th_1 response over a Th_2 response is a functional property of 3-DMPL, applicants respectfully request that this Honorable Board also disregard this statement as improper under established Patent Law principles and also as an incorrect

representation of the state of the art. The following references indicate that MPL has different T cell responses when exposed to different antigens. *See, e.g.*, Johansen et al., *Immunogenicity and Protective Efficacy of a Formalin-Inactivated Rotavirus Vaccine Combined with Lipid Adjuvants*, VACCINE 21(5-6):368-375 (2003) (Balb/c mice immunized with rotavirus vaccine with MPL demonstrate a 1:1 ratio of IgG_1 and IgG_{2a} suggesting a balanced Th_1/Th_2 response) (attached at Appendix D); and Yang et al., *Mechanisms of Monophosphoryl Lipid A Augmentation of Host Responses to Recombinant HagB from Porphyromonas Gingivalis*, INFECTION AND IMMUNITY 70(7):3557-3565 (2002) (Balb/c mice injected with recombinant hemagglutinin B (rHagB) with and without MPL provided evidence that MPL potentiates a Th_2 response to HagB) (attached at Appendix E). These references indicate that there are exceptions to the inducing effects of MPL.

Turning to the issue of the Examiner's selection of the WO 92/16556 reference as a secondary reference, applicants submit that this reference constitutes nonanalogous art and should not be the subject of an obviousness rejection over the claimed invention. Applying the two-step test from *In re Deminski, supra*, the nonanologous nature of the reference become clear: (i) the WO 92/16556 reference, which addresses the treatment of HIV/AIDS, is clearly not within the applicants' field of endeavor of the treatment of allergies; and (ii) the WO 92/16556 reference, which relates to the production of a vaccine for HIV/AIDS using a single glycoprotein of a viral antigen combined with 3-DMPL, does not bear a reasonable relation to the allergy desensitization therapy with which the applicants' are involved. In light of the nonanologous nature of the WO 92/16556 reference, the use of 3-DMPL as an adjuvant in the vaccine disclosed in the WO 92/16556 reference cannot serve to save this reference from its irrelevance.

The foregoing discussion demonstrates the many flaws in the Examiner's hypothetic combination of WO 96/34656 in view of WO 92/16556. Bearing these flaws in mind, the Examiner's obviousness rejection falls apart quickly.

Recapping the deficiencies of the primary reference, WO 96/34656 fails to teach or suggest the use of 3-DMPL in the modified allergen-tyrosine formulation disclosed therein and also fails to teach or suggest that the enhancement of a Th_1 response over a Th_2 response may increase desensitization therapy for allergy suffers.

As mentioned above, the WO 92/16556 reference constitutes nonanalogous art and therefore should not be combined with the primary reference. Even assuming *arguendo* that WO 92/16556 has a place in the Examiner's obviousness rejection, this reference fails to correct the deficiencies of WO 96/34656 for the following significant reasons.

First, WO 92/16556 does ***not*** provide the missing teaching from the primary reference that the enhancement of a Th_1 response over a Th_2 response may increase desensitization of a patient that is

suffering from an immune response; accordingly, the ordinary artisan could not have a reasonable expectation of arriving at the claimed invention merely by applying the 3-DMPL from WO 92/16556 to the composition disclosed in WO 96/34656.

Second, because WO 96/34626 does not teach or suggest that desensitization therapy may be made more effective if a Th_1 response is enhanced over a Th_2, the ordinary artisan has no motivation to turn to any reference that discloses a method by which a Th_1 response is enhanced over a Th_2 response. Accordingly, even if the 3-DMPL of the WO 92/16556 reference did enhance a Th_1 over a Th_2 response, the ordinary artisan would have no motivation to seek out the 3-DMPL of the reference because the WO 96/34656 reference provides no suggestion that this is desirable. In light of the lack of motivation provided in the primary WO 96/34656 reference for the ordinary artisan to turn to the 3-DMPL adjuvant to enhance a Th_1 response over a Th_2 response, applicants can only conclude that the Examiner's use of references disclosing 3-DMPL, as well as the Examiner's arguments that an enhancement of a Th_1 response over a Th_2 response is an inherent function 3-DMPL (which was shown above not to be an incorrect statement as 3-DMPL induces different T cell responses when exposed to different antigens), are the result of an impermissible hindsight reconstruction of the present invention from the disclosure of the instant application. *See*, *In re Deuel*, *supra*. That the Examiner misinterpreted the disclosure is immaterial, a hindsight reconstruction, whether based on a correct or incorrect interpretation of the art, is *impermissible* under established Patent Law principles.

On the issue of the Examiner's hindsight reconstruction, applicants emphasize that the Examiner could have never applied the WO 92/16556 reference to the claimed invention without the benefit of the applicants' disclosure that the enhancement of a Th_1 response over a Th_2 response is effective to increase desensitization therapy in allergy sufferers. *See*, *In re Oetiker*, *supra*. Without this knowledge, the WO 92/16556 reference would seem like nothing more than an irrelevant reference in a nonanalogous art that happens to use 3-DMPL as an adjuvant in anti-viral therapy for HIV/AIDS; however, with the benefit of applicants' invention, the Examiner is able to assign a purpose to the 3-DMPL in WO 92/16556. Such an application of the invention is of course a clear violation of the prohibition against hindsight reconstruction.

The last cited reference, the '862 Patent, teaches a formulation and method for isolating ectoparasite saliva proteins and a composition for detecting allergic dermatitis in an animal (col. 1, ll. 15-17). The composition of the '862 Patent is disclosed as including "Ribi adjuvant," which may serve as a carrier to enhance the immune response of an animal to a specific antigen (col. 42, ll. 19-25 and 32). Not only is there ***no*** indication in this reference that the "Ribi adjuvant" is 3-DMPL, there is also ***no*** disclosure in this reference on how or in what way the adjuvant increases the immune response of the

animal. Notwithstanding the foregoing, even if it were generally known in the art that Ribi adjuvant is 3-DMPL, as the Examiner asserts, without some general suggestion from the WO 96/34626 reference for the ordinary artisan to seek out an adjuvant to add to the composition of WO 96/34626, the ordinary artisan would have no motivation to turn to the '862 Patent to include the Ribi adjuvant disclosed therein.

At the second paragraph of page 14 of the Examiner's Answer, the Examiner asserts that the Ribi adjuvant of the '862 is known to be 3-DMPL because the WO 92/16556 reference teaches that the 3-DMPL disclosed therein is from Ribi. These two teachings in combination, however, do not serve to save the present invention, as mentioned above, without the proper motivation from the WO 96/34626 reference to seek out an adjuvant for the enhancement of a Th_1 response over a Th_2 response, an isolated use of 3-DMPL in a prior art reference remains meaningless. *See*, *In re Rouffet*, *supra*.

In response to the Examiner's assertion that it would be obvious to add the Ribi adjuvant from the '862 Patent to the modified allergen and tyrosine combination of WO 96/3626 because both reference disclose art useful for the same purpose (the Examiner cites *In re Kerkhoven*, 626 F.2d 846, 205 USPQ 1069 (CCPA 1980) to support this position), applicants submit that even if this were the case, the ordinary artisan would not have a reasonable expectation that the Ribi Adjuvant would enhance a Th_1 over a Th_2 response because there is nothing in the primary reference to suggest that an adjuvant will achieve that end. That the '862 Patent suggests that an adjuvant may enhance the immune response of an animal to a specific antigen (col. 42, ll. 23-25) is not a sufficient disclosure for the ordinary artisan to leap to the conclusion that the application of an adjuvant to the composition of WO 96/34626 will enhance a Th_1 response over a Th_2 response. Accordingly, applicants submit that contrary to the Examiner's assertion, the application of the Ribi adjuvant of the '862 Patent to the composition of the WO 96/34626 reference will not render the claimed invention obvious.

The foregoing discussion demonstrates that the Examiner's obviousness rejection fails because it applies hindsight reconstruction to arrive at the claimed invention. The Examiner asserts that the WO 92/16556 reference and the '862 Patent teach the use of 3-DMPL; however, without the proper motivation from the primary WO 96/34626 reference for the ordinary artisan to turn to these references, the teachings set forth therein are meaningless. Furthermore, despite the Examiner's best attempts to read the enhancement of a Th_1 response over a Th_2 response into the disclosures of 3-DMPL in the cited references, the foregoing discussion shows that the Examiner's attempts are nothing more than an impermissible hindsight reconstruction disguised as common knowledge.

Because the Examiner's hypothetical combination of WO 96/34656 in view of WO 92/16556 and the '865 Patent does not serve to render the claimed invention obvious, applicants respectfully request that this Honorable Board reverse the Examiner's final rejection of claims 1, 2, 6-8, 15-17, and 19-23.

C. CLAIM 18 IS NOT OBVIOUS OVER WO 96/34626 IN VIEW OF WO 92/16556 AND THE '862 PATENT AS APPLIED TO CLAIMS 1, 2, 6-8, 15-17, AND 19-23 AND FURTHER IN VIEW OF MARSH, THE '110 PATENT, AND HOYNE ET AL.

Claim 18, which depends from claim 1, recites an embodiment of the claimed invention wherein the allergen extract is not modified by reaction with a cross-linking agent.

Because claim 1 is not rendered obvious by the combination of the WO 96/34656 reference in view of the WO 92/16556 reference and the '862 Patent as set forth above, it follows that claim 18 cannot be rendered obvious by the cited combination of references.

Notwithstanding the foregoing, applicants are discussing the merits of this rejection solely for the purpose of demonstrating the irrelevance the Examiner's citation of the '110 Patent and Hoyne et al. to the invention *as claimed in claim 18.*

The Examiner cites Marsh for the teaching of an unmodified allergen and a chemically modified allergen such as formalinized allergen. As already noted, this teaching will not serve to obviate the patentability of claim 18 due to its dependency on claim 1.

As described above, the '110 Patent teaches a vaccine formulation that includes 3-DMPL and QS21 (a saponin derivative) for the treatment of various viral diseases, such as immunodeficiency viruses, herpes viruses, cytomegalovirus, varicella zoster virus, hepatitis virus, respiratory sunctial virus, human papilloma virus, influenza virus; bacterial disesases, such as salmonella, neisseria, borellia, Chlamydia, bordetella; or parasitic diseases, such as plasmodium or toxoplasma (col. 1, ll. 56-57). The '110 Patent does ***not*** contemplate applying the vaccine to allergens and thus, there is no mention in the '110 Patent of modified or unmodified antigens.

Hoyne et al. is a review paper that discloses that desensitized allergy patients have decreased Th_2 cells and postulates that immune responses may be reprogrammed by promoting Th_1 responses *instead* of Th_2 responses (Abstract). There are two references to allergens in Hoyne et al.: one is at page 181 (1^{st} col.) where ovalbumin is mentioned and the other is at page 183 (top of 1^{st} full para.), where multideterminate allergens such as house dust mite allergy are mentioned. Hoyne et al. does not, however, discuss if the allergens are modified or unmodified, which is not surprising as the emphasis of Hoyne et al. is clearly the T cell responses to allergens rather than the structure or the source of the allergens themselves.

Applicants are baffled as to the reason these two references are included in this rejection, which is directed solely to claim 18, and do not understand the Examiner's motivation for applying the teachings of the '110 Patent and Hoyne et al. to the features of claim 1, when it is clear from the nature of the rejection that the Examiner is applying WO 96/34626 in view of WO 92/16556 and the '862 Patent to claims 1, 2, 6-8, 15-17, and 19-23, as discussed in the previous section, and is applying Marsh, the '110

Patent, and Hoyne et al. to *claim 18.* Had the Examiner wished to apply the '110 Patent and Hoyne et al. to claim 1, then the Examiner should have characterized this rejection as including the '110 Patent and Hoyne et al. over claim 1. Because the Examiner did not do this, applicants respectfully request that this Honorable Board disregard the Examiner's application of the '110 Patent and Hoyne et al. to claim 1 and consider these references only insofar as they apply to claim 18.

Solely for the purpose of ensuring that applicants failure to address the Examiner's application of the '110 Patent and Hoyne et al. to claim 1 is ***not*** interpreted as an acquiescence to the Examiner's application of the references as such, applicants will discuss the '110 Patent and Hoyne et al. as if they have been added to the combination of WO 96/34626 in view of WO 92/16556 and the '862 Patent.

At page 7 of the Examiner's Answer (3rd line from the top), the Examiner makes the bald statement that the '110 Patent teaches a Th_1 response over a Th_2 response. This is clearly not the case. *Nowhere* in the '110 Patent is it taught that a Th_1 response is enhanced over a Th_2 response for any reason. The Examiner makes this reckless assertion based upon the teaching in the '110 Patent that 3-DMPL and QS21, in combination, and in lesser form alone, enhance immune response. In the 110 Patent, at col. 1, line 26, and at Example 2.4 (beginning at col. 6, l.33), the induction of CTLs are disclosed as one immune response and at col. 2, lines 40-41, and at Example 1.3 (col. 5, l.1) IFN-γ secreted from Th_1 cells is disclosed as another immune response. Accordingly, the '110 Patent teaches only that 3-DMPL, synergistically with QS21 and possibly alone, may enhance immune responses by through the induction of CTLs and/or through increased secretion of IFN-γ, the latter via Th_1 cells. This teaching is far from asserting that 3-DMPL enhances a Th_1 response over a Th_2 response.

When the teachings of the '110 Patent are combined with the teachings of the references cited against claim 1, the '110 Patent still fails to correct the deficiencies of WO 96/34626 in view of WO 92/16556 and the '862 Patent.

To recap, the WO 96/34626 in view of WO 92/16556 and the '862 Patent are deficient because the primary reference, WO 96/34626, fails to provide any motivation for the ordinary artisan to seek out an adjuvant, and even if the adjuvants disclosed in the secondary and tertiary references were applied to the modified allergen and tyrosine combination of WO 96/34626, there would be no reasonable expectation that the adjuvants would enhance a Th_1 response over a Th_2 response.

Under this rubric, applicants submit that because WO 96/34626 lacks a motivation for the ordinary artisan to seek out an adjuvant, the ordinary artisan would have no reason to combine the '110 Patent with the primary WO 96/34626 reference; however, and again solely for the sake of argument, were the ordinary artisan to apply the 3-DMPL of the '110 Patent to the composition of WO 96/34626 in view of WO 92/16556 and the '862 Patent, the most that the ordinary artisan could reasonably expect

from the combination would be increased IFN-γ secretion from Th_1 cells. Applicants submit that an increase in a Th_1 response is ***not*** tantamount to an enhancement of a Th_1 response over a Th_2 response. In other words, although the 3-DMPL may increase a Th_1 response (in the form of increased IFN-γ), under the teachings of WO 96/34626 in view of WO 92/16556, the '862 Patent, and the '110 Patent, the increased Th_1 response may still be less than or equal to the Th_2 response.

The Examiner asserts that Hoyne et al. teach that "a major key to successful immunotherapy depends on reprogramming the immune response toward Th_1 because decreasing the functional response of Th_2 cells would have been expected to improve clinical symptoms" (Ex. Answ., p.7, 1st full para.). Applicants note that Hoyne et al. do ***not*** teach what the Examiner asserts; Hoyne et al. teach the follow observation:

> Patients who have been desensitized normally display a decrease in Th_2 cytokine production, and clinical improvement usually correlates with a decrease in immediate and late phase skin reactivity with a long-term rise in IgG levels, particularly IgG_4, and a decrease in specific IgE (p.183, col. 1, ¶ 2).

From this observation, Hoyne et al. *postulate* that successful immunotherapy may depend on altering the qualitative nature of the Th response by administering mucosally delivered antigens to *functionally silence memory Th_2/Th_0 cells.* With the silencing of the Th_2 cells, Hoyne et al. suggest that it *may* be possible to reprogram the immune response towards a Th_1 phenotype.

While it is undoubtful that the Examiner reads this disclosure to mean that Hoyne et al. teach the selective enhancement of a Th_1 response over a Th_2 response, applicants reiterate that Hoyne et al. do not teach this; Hoyne et al. teach the *functional silencing of Th_2 cells* and the *switching of the immune response to Th_1 cells*. With Hoyne et al. it is an either-or proposition.

Thus, when applying Hoyne et al. to the hypothetical combination of WO 96/34626 in view of WO 92/16556, the '862 Patent, and the '110 Patent, it must first be kept in mind that applicants maintain that the primary reference, WO 96/34626, provides ***no*** motivation for the ordinary artisan to turn to any of the secondary references. Applicants concede, however, that given the teaching of desensitization therapy in Hoyne et al., the ordinary artisan, at the time of the invention, would probably have been tempted to peruse Hoyne et al. to see what it may add to the teachings of WO 96/34626. Having done so, the ordinary artisan would obviously glean the teaching from Hoyne et al. *that the desensitization effects of the allergen-tyrosine composition of WO 96/34626 are due to a decrease in Th_2 production* and that the desensitization effects *could be further decreased by functionally silencing* the Th_2 cells in the allergic patient.

Because Hoyne et al. suggest that an increase in IFN-γ production is correlated with a decrease in Th_2 response (p.180, para. bridging col. 1 to col. 2), logically, the ordinary artisan would be likely to search for a composition that would increase IFN-γ production in the patient to the point where the patient's Th_2 cells would be *functionally silenced.* Here, the Examiner's '110 Patent may find application with its teaching that the combined adjuvants 3-DMPL and QS21 increase IFN-γ secretion from Th_1 cell; however, in light of the substantially reduced effect of 3-DMPL and QS21 alone in enhancing IFN-γ secretion ('110 Patent, Example 1.3), the ordinary artisan would not be motivated to apply either adjuvant alone. Notwithstanding the foregoing, because Hoyne et al. teach that desensitization therapy results from a decreased response in Th_2 cells (which would be inherent in the desensitization therapy of WO 96/34626) and suggests the functional silencing of Th_2 cells may improve desensitization therapy, the teaching in the '110 Patent that 3-DMPL and QS21 enhances IFN-γ secretion will only serve to provide the ordinary artisan with a vehicle to functionally silence the Th_2 cells by forcing the Th_1 cells to produce more IFN-γ.

Because functionally silencing Th_2 cells with 3-DMPL and QS21 is not the same as selectively enhancing a Th_1 response over a Th_2 response with 3-DMPL, applicants submit that even if the Examiner had applied the hypothetical combination of WO 96/34626 in view of WO 92/16556, the '862 Patent, the '110 Patent, and Hoyne et al. over the invention as recited in claim 1, the rejection would ***not*** have rendered the claimed invention obvious.

At this time, applicants must remind this Honorable Board that because the Examiner's rejection *only refers to claim 18*, the foregoing analysis is completely irrelevant to the issues before the Board and as previously stated serves *only* to demonstrate applicants' good faith in parsing the Examiner's rejections to ensure that all aspects of the rejection have been addressed, both literally and implicitly.

Returning to the issue before the Board, which is the patentability of claim 18, applicants have already stated the obvious conclusion that dependent claim 18 is not rendered obvious by the hypothetical combination of WO 96/34656 in view of WO 92/16556, and the '862 Patent as applied to claim 1 and further in view of Marsh. As discussed already, the '110 Patent and Hoyne et al. do not discuss the modification of unmodification of allergens and thus, add nothing to the Marsh reference that could be applied against claim 18. In light of the foregoing, it follows that claim 18 is not rendered obvious by the hypothetical combination of WO 96/34656 in view of WO 92/16556, and the '862 Patent as applied to claim 1 and further in view of Marsh, the '110 Patent, and Hoyne et al.; accordingly, applicants respectfully request that this Honorable Board reverse the Examiner's final rejection of claim 18.

D. CLAIMS 1 AND 23 ARE NOT OBVIOUS OVER WO 96/34626 IN VIEW OF HOLEN ET AL., WO 92/16556, THE '110 PATENT, AND HOYNE ET AL.

Claim 23, which depends from claim 1, recites an embodiment of the claimed invention wherein the allergen or the allergen extract is selected from the group consisting of grass pollen and ovalbumin. Because claim 23 depends from claim 1, the cited references, alone or in combination must teach or suggest all of the elements of claim 1 plus at least on of the additional elements of claim 23 in order for the Examiner's obviousness rejection to stand.

The Examiner cites the WO 96/34626 reference as the primary reference; this reference was already discussed in detail in this Reply Brief and needs no further elaboration. With respect to the secondary references, the Examiner cites Holen et al. in view of WO 92/16556, the '110 Patent, and Hoyne et al. This rejection baffles applicants because the order of the references appears to be completely random. For example, on page 8 of the Examiner's Answer, after discussing the WO 96/34626 reference, which is obviously directed to claim 1, the Examiner discusses Holen et al., which given its subject matter must be directed to claim 23, but then the discussion resumes with the WO 92/16556 reference, the '110 Patent, and Hoyne et al., which are clearly directed to claim 1 again. For the sake of clarity then, applicants must treat this rejection as if it is two separate rejections: (1) a rejection directed to claim 1 over WO 96/34626 in view of WO 92/16556, the '110 Patent, and Hoyne et al., and (2) a rejection directed to claim 23 over WO 96/34626 in view of WO 92/16556, the '110 Patent, and Hoyne et al. as applied to claim 1 and further in view of Holen et al.

The combination of WO 96/34626 in view of WO 92/16556, the '862 Patent, the '110 Patent and Hoyne et al. was discussed in the previous section. The omission of the '862 Patent from this rejection does not warrant an additional discussion because as already explained, the '862 Patent only discloses the general use of Ribi Adjuvant to enhance the immune response of animals to a specific antigen and thus, does not add anything of significance to the teachings of the other references. The Examiner cites Holen et al. for the teaching of ovalbumin as an allergen.

As stated in the previous section, because the hypothetical combination of WO 96/34656 in view WO 92/16556, the '862 Patent, the '110 Patent and Hoyne et al. teaches the functional silencing of Th_2 cells from allergic patients with a combination of 3-DMPL and QS21; rather than the selective enhancement of a Th_1 response over a Th_2 response, the hypothetical combination does not render the invention as recited in claim 1 obvious. Because claim 1 is not rendered obvious by the combination of WO 96/34656 in view of WO 92/16556, the '862 Patent, the '110 Patent, and Hoyne et al. as set forth above, it follows that the additional teaching of Holen et al. cannot serve to render claim 23 obvious;

accordingly, applicants respectfully request that this Honorable Board reverse the Examiner's final rejection of claims 1 and 23.

CONCLUSION:

With this appeal, applicants respectfully request the following actions from this Honorable Board. First, applicants request that this Honorable Board disqualify the WO 96/34626 reference as prior art for this application under 35 U.S.C. § 103(a) because the date of invention of the claimed subject matter predates the effective date of the WO 96/34626 reference. Second, applicants request that this Honorable Board reverse the Examiner's three obviousness rejections on the grounds that none of the cited references, whether qualifying or not, and whether applied alone or in combination, teach or suggest the claimed invention.

Because applicants have demonstrated in this Reply Brief, and the Appeal Brief filed before it, that the Examiner has failed to establish a *prima facie* case of unpatentability against the claimed invention, applicants submit that they are entitled to a patent grant on the claimed inventin.

Respectfully submitted,

By: Karen Canaan

Karen Canaan
Registration No. 42,382

REED & EBERLE LLP
800 Menlo Avenue, Suite 210
Menlo Park, California 94025
(650) 330-0900 Telephone
(650) 330-0980 Facsimile

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<u>Claims on Appeal</u>

1. **(previously amended)** A pharmaceutical composition capable of selectively enhancing a TH_1 response over a TH_2 response, comprising tyrosine, an allergen or allergen extract, and 3-DMPL.

2. **(previously amended)** A composition according to claim 1, wherein the allergen or allergen extract is coated with and/or adsorbed onto tyrosine.

6. **(previously amended)** A composition according to claim 2, wherein the allergen or allergen extract is coated with the tyrosine.

7. **(previously amended)** A composition according to claim 2, wherein the allergen or allergen extract is adsorbed onto the tyrosine.

8. **(previously amended)** A composition according to claim 2, wherein the allergen or allergen extract is coated with and adsorbed onto the tyrosine.

15. **(previously added)** A composition according to claim 1, wherein the allergen or allergen extract is modified by reaction with a cross-linking agent.

16. **(previously added)** A composition according to claim 15, wherein the cross-linking agent is a dialdehyde.

17. **(previously added)** A composition according to claim 16, wherein the dialdehyde is glutaraldehyde.

18. **(previously added)** A composition according to claim 1, wherein the allergen or the allergen extract is not modified by reaction with a cross-linking agent.

19. **(previously added)** A composition according to claim 1, wherein the allergen or the allergen extract is derived from a source selected from pollen, food, insect venom, mold, animal fur, house dust mite, and combinations thereof.

20. **(previously added)** A composition according to claim 19, wherein the allergen or the allergen extract is derived from ragweed pollen or birch pollen.

21. **(previously added)** A composition according to claim 19, wherein the allergen or allergen extract is derived from dust mite of species *D. farinae* or *D. pteryssinus.*

22. **(previously added)** A composition according to claim 19, wherein the allergen or the allergen extract is selected from the group consisting of pollen and food.

23. **(previously added)** A composition according to claim 22, wherein the allergen or the allergen extract is selected from the group consisting of grass pollen and ovalbumin.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In Re Application of:
Jorj Terry ULRICH et al.

Confirmation No. 5731

Serial No. 09/402,273

Group Art Unit 1644

Filing Date: December 13, 1999

Examiner Phuong N. HUNYH

Title: ALLERGEN FORMULATION

DECLARATION OF KAREN CANAAN IN SUPPORT OF THE SUBMISSION OF THE DECLARATION OF ALAN WORLAND WHEELER UNDER 37 C.F.R. § 1.131

I, Karen Canaan, declare:

1. I am an attorney of record for the above-identified patent application, identified by U.S. Patent Application Serial No. 09/402,273 (hereinafter referred to as the "instant application"). In my capacity as an attorney of record for the instant application, I prepared the Appeal Brief, filed on September 12, 2003, and the Reply Brief, filed on July 6, 2004.

2. In the Appeal Brief, filed on September 12, 2003, the Declaration of Alan Worland Wheeler was attached to the Brief as evidence of a date of invention prior to the November 7, 1996, effective date of the WO 96/34626 reference. Dr. Wheeler is one of the two inventors of the subject matter described in the instant application and Dr. Jorg Terry Ulrich is Dr. Wheeler's co-inventor. At the time that the Declaration of Alan Worland Wheeler was prepared, Dr. Ulrich could not be located; therefore, the Declaration of Alan Worland Wheeler was not accompanied by a Declaration of his co-inventor as required under 37 C.F.R. § 1.131. It was my intention at the time the Appeal Brief was filed to continue to try to locate Dr. Ulrich and to submit his Declaration with the Reply Brief.

3. Prior to filing the Appeal Brief, I researched Dr. Ulrich's most recent place of employment as Corixa Corporation in Hamilton, Montana; however, upon contacting Corixa Corporation, I was informed that Dr. Ulrich no longer worked at Corixa Corporation and had left the corporation approximately two years prior, which would be approximately 2002. Corixa informed also me that they did not have a forwarding address for Dr. Ulrich. At that time, I conducted numerous on-line searches using available free on-line people searching databases, but was unable to locate anyone that could definitively be the Dr. Jorg Terry Ulrich that I was looking for. I could not locate anyone named "Jorg Terry Ulrich" or "J. Terry Ulrich" anywhere in the country. Searches for "Terry Ulrich" and "T. Ulrich" throughout the country resulted in far too many listings to be of use.

4. Upon preparing this Reply Brief, I again started to search for Dr. Ulrich. To do so, on June 29, 2004, I contacted Corixa Corporation one more time and inquired if anyone there knew Dr. Ulrich's whereabouts. On that day, I spoke with a receptionist who remembered Dr. Ulrich; she informed me that she did not have a forwarding address for Dr. Ulrich, but offered to search the current Montana phone book in paper copy for his listing. Upon doing so, she informed me that he was not listed there.

5. On July 2, 2004, I contacted Corixa Corporation one more time to see if there was anyone at the corporation who could remember Dr. Ulrich, and I was transferred to Human Resources. As noone answered the phone, I left a message for the Director of Human Resources at Corixa Corporation identifying myself as a patent attorney prosecuting a patent application that has Corixa's former employee, Dr. Jorg Terry Ulrich, as an inventor and inquiring if Corixa Human Resources had a forwarding address or telephone number that they could provide to me. As of the date of this Declaration, I had not received a return phone call from Corixa Corporation.

6. Having had no luck with my communications with Corixa Corporation, I again searched numerous free on-line people searching databases for "Jorg Terry Ulrich" and "J. Terry Ulrich," but again could not locate any such person anywhere in the U.S. I then tried searches for "Terry Ulrich" in Montana and nationally, but was unable to confirm that any of the names were the person that I am looking for. To narrow my search, I conducted a $10.00 people search at "www.privateeye.com" for "J. Terry Ulrich" in Montana, and located one person in Corvallis, Montana, which is close to Hamilton, Montana, where Corixa Corporation is located. The

www.privateeye.com search for "J. Terry Ulrich" in Montana; the contact page for Corixa Corporation, showing its address in Hamilton, Montana; and a www.mapquest.com print-out for Corvallis, Montana, showing its proximity to Hamilton, Montana, are all attached to this Declaration.

7. Having located a "J. Terry Ulrich" near Hamilton, Montana, I telephoned the number identified for this person on or about 2:00 p.m. on July 2, 2004, and an voice recording service message picked up the call, which had a woman's voice; she did not identify herself as "J. Terry Ulrich." The woman's message, however, stated: "I cannot come to the phone right now...," which appears to indicate that she is probably the "J. Terry Ulrich" identified in the search. Because Dr. Ulrich, the co-inventor for this application is a man, the voice on the voice recording service message leads me to believe that I did not contact the correct person. Nevertheless, I left a message for the J. Terry Ulrich that I contacted identifying myself as a patent attorney prosecuting a patent application that has a Jorg Terry Ulrich as an inventor with a last known location in or around Hamilton, Montana and requested a return phone call if I had contacted the correct person. I also apologized if I did not contact the correct person and requested a courtesy call to confirm that I am in fact mistaken. As of July 5, 2004, I had not received a call from the person that I had contacted.

8. Immediately after contacting the J. Terry Ulrich in Hamilton, Montana, I conducted another $10.00 search on www.privateeye.com for a "J. Terry Ulrich" on a nationwide search. This nationwide search is also attached to this Declaration. On this nationwide search, the "J. Terry Ulrich" in Corvallis, Montana that I had just contacted is the first entry, identified as a "J.T. Ulrich," and a second "J.T. Ulrich" in Philadelphia Pennsylvania is the second entry. I contacted the J.T. Ulrich in Philadelphia on or about 3:00 p.m. on July 2, 2004, but noone answered the phone after more than ten rings. I tried this number at least three more times between July 2, 2004, and July 5, 2004, but the phone never picked up, it just kept on ringing.

9. Because I have been unable to locate Dr. Jorg Terry Ulrich through any available means, I am requesting that the Office accept the Declaration of Alan Worland Wheeler as the sole available inventor for this application on the grounds that co-inventor Dr. Jorg Terry Ulrich cannot be reached after diligent efforts to do so.

10. All statements made herein of my own knowledge are true and all statements made herein on information and belief are believed to be true; further, all statements made herein were made with the full knowledge that willful false statements are punishable by fine, imprisonment, or both under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the instant application or any patent issuing thereon.

Dated: July 6, 2004

Karen Canaan

Karen Canaan

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	Full Name	Address	Age	Birth Date	Phone	
1.	ULRICH, J TERRY	883 HAMILTON HEIGHTS RD CORVALLIS, MT 59828				View Detai
2.	ULRICH, J T	883 HAMILTON HEIGHTS RD CORVALLIS, MT 59828			(406) 961-4196	View Detai
3.	ULRICH, J T	883 HAMILTON HEIGHTS CORVALLIS, MT 59828			(406) 961-4196	View Detai
4.	ULRICH, J T	883 HAMILTON HEIGHTS RD CORVALLIS, MT 59828				View Detai
5.	ULRICH, J T	977 HAMILTON HEIGHTS CORVALLIS, MT 59828			(406) 961-4196	View Detai
6.	ULRICH, JUSTIN A	325 SANDY HILL LN COLUMBIA FALLS, MT 59912				View Detai
7.	ULRICH, JUSTIN A	325 SANDY HILL LN COLUMBIA FALLS, MT 59912				View Detai

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cancer | infectious disease | autoimmune disease

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collaborations
management
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contacts

contacts

Corporate Headquarters
1124 Columbia Street
Suite 200
Seattle, WA 98104
Phone: 206.754.5711
Fax: 206.754.5715
info@corixa.com

South San Francisco Office
600 Gateway Boulevard
South San Francisco, CA 94080
Phone: 650.553.2000
Fax: 650.553.2028

Manufacturing and Adjuvant Development
553 Old Corvallis Road
Hamilton, MT 59840
Phone: 406.363.6214
Fax: 406.363.6129

BEXXAR Service Center
1.877.4BEXXAR

Clinical Affairs
clinicalaffairs@corixa.com

Human Resources
employment@corixa.com

Investor Relations
investor.relations@corixa.com

Medical Affairs
Drug Safety (Adverse Events) and Medical Information
medicalaffairs@corixa.com

Partnering/Business Development
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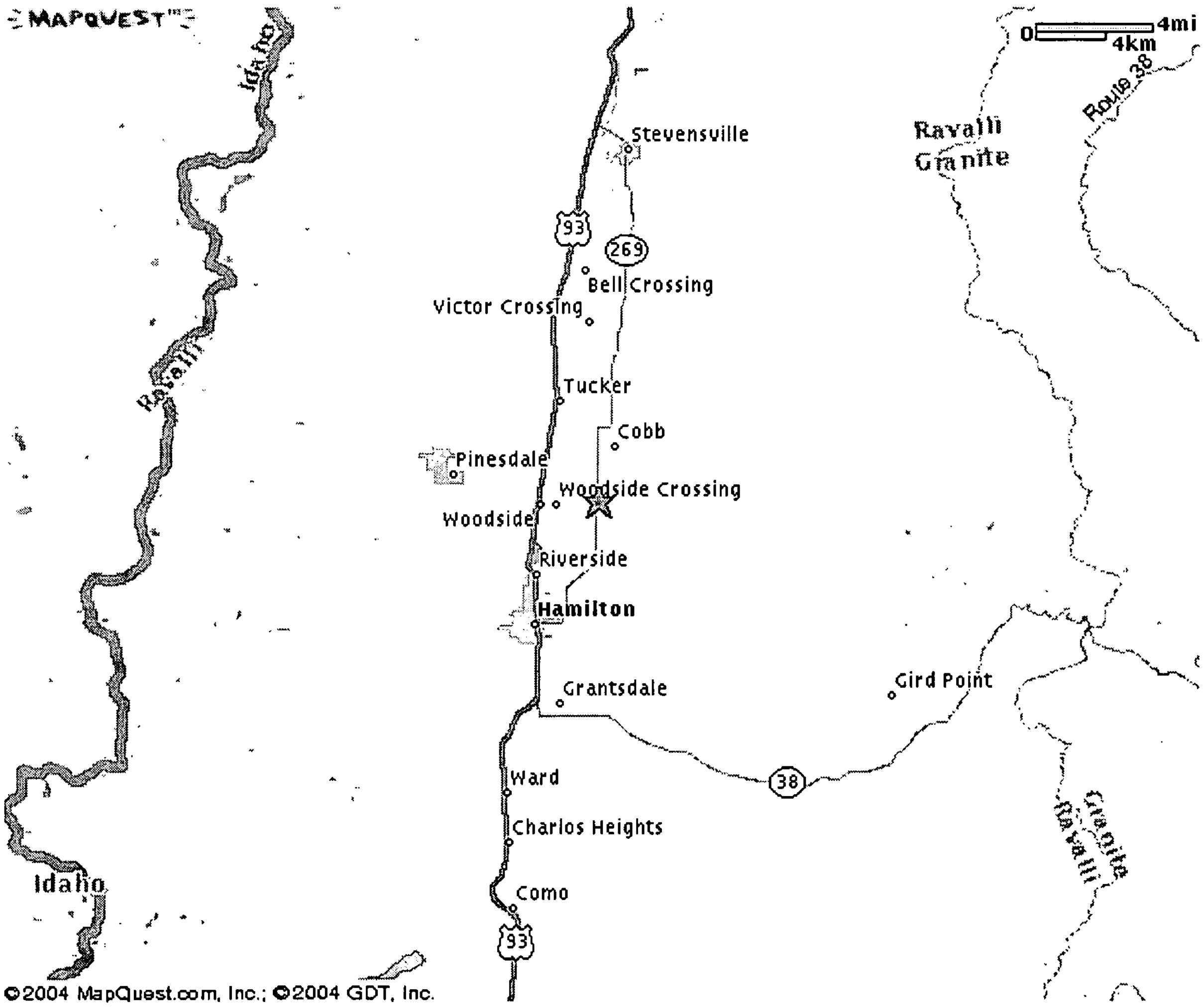

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	Full Name	Address	Age	Birth Date	Phone	
1.	ULRICH, J T	883 HAMILTON HEIGHTS RD CORVALLIS, MT 59828			(406) 961-4196	Vie
2.	ULRICH, J T	4018 KENSINGTON AVE PHILADELPHIA, PA 19124			(215) 533-1807	Vie
3.	ULRICH, JAMES T	404 SYCAMORE RD READING, PA 19611	53	12/22/1950	(484) 678-6744	Vie
4.	ULRICH, JAMES T	20 RR 1 ROBESONIA, PA 19551	53	12/22/1950	(610) 678-6744	Vie
5.	ULRICH, JAMES T	FOXCROFT LN ROBESONIA, PA 19551	53	12/22/1950	(610) 678-6744	Vie
6.	ULRICH, JAMES T	271 WALTERS AVE WERNERSVILLE, PA 19565	53	12/22/1950	(610) 678-6744	Vie
7.	ULRICH, JAMES T	404 SYCAMORE RD WEST READING, PA 19611	53	12/22/1950	(484) 678-6744	Vie
8.	ULRICH, JAMES T	555 MAINE AVE LONG BEACH, CA 90802	55	10/15/1948	(562) 320-4816	Vie
9.	ULRICH, JAMES T	27558 EASTVALE RD PALOS VERDES PENINSULA, CA 90274	55	10/15/1948	(310) 544-2003	Vie
10.	ULRICH, JAMES T	3117 SINGINGWOOD DR TORRANCE, CA 90505	55	10/15/1948	(310) 544-2003	Vie
11.	ULRICH, JAMES T	3177 SINGINGWOOD DR TORRANCE, CA 90505	55	10/15/1948	(310) 544-2003	Vie
12.	ULRICH, JAMES T	203 N MUSQUEQUAK ST BURLINGTON, WI 53105	58	07/14/1946	(262) 662-4482	Vie
13.	ULRICH, JAMES T	403 MARYL ST BURLINGTON, WI 53105	58	07/14/1946	(262) 662-4482	Vie
14.	ULRICH, JAMES T	11920 W JANESVILLE RD HALES CORNERS, WI 53130	58	07/14/1946	(414) 662-4482	Vie
15.	ULRICH, JAMES T	2427 S 95TH ST MILWAUKEE, WI 53227	58	07/14/1946	(414) 662-4482	Vie
16.	ULRICH, JAMES T	3161 S 106TH ST MILWAUKEE, WI 53227	58	07/14/1946	(414) 662-4482	Vie
17.	ULRICH, JAMES T	8315 HALVERSON RD WATERFORD, WI 53185	58	07/14/1946	(262) 662-4482	Vie
18.	ULRICH, JAMES T	RR CARMEL, NY 10521	60	05/1944	(914) 277-5463	Vie
19.	ULRICH, JAMES T	1 SHINDAGEN RD	60	05/1944	(914) 277-5463	Vie

		CROTON FALLS, NY 10519				
20.	ULRICH, JAMES T	534 PO BOX CROTON FALLS, NY 10519	60	05/1944	(914) 277-5463	Vie
21.	ULRICH, JAMES T	2276 6TH ST EAST MEADOW, NY 11554	60	05/1944		Vie
22.	ULRICH, JAMES T	271 WALTERS AVE WERNERSVILLE, PA 19565			(610) 678-6744	Vie
23.	ULRICH, JAMIE T	105 COLONIAL DR AKRON, PA 17501	40	10/10/1963	(717) 859-3332	Vie
24.	ULRICH, JAMIE T	103 MILLSTONE DR DENVER, PA 17517	40	10/10/1963	(717) 445-0353	Vie
25.	ULRICH, JAMIE T	185 BRICKYARD CIR EPHRATA, PA 17522	40	10/10/1963	(717) 859-3332	Vie
26.	ULRICH, JAMIE T	861 PO BOX EPHRATA, PA 17522	40	10/10/1963	(717) 859-3332	Vie
27.	ULRICH, JAMIE T	13 SYCAMORE CIR STEVENS, PA 17578	40	10/10/1963	(717) 859-3332	Vie
28.	ULRICH, JANELL T	1761 CRYSTAL WAY PLANO, TX 75074	58	03/15/1946	(972) 424-5468	Vie
29.	ULRICH, JANELL T	2308 DYERS OAK DR PLANO, TX 75074	58	03/15/1946	(972) 424-5468	Vie
30.	ULRICH, JANELL T	2308 DYERS OAK DR PLANO, TX 75074	58	03/15/1946	(972) 424-5468	Vie
31.	ULRICH, JANELL T	2803 DYERS OAK PLANO, TX 75074	58	03/15/1946	(972) 424-5468	Vie
32.	ULRICH, JANELL T	2803 DYERS OAK DR PLANO, TX 75074	58	03/15/1946	(972) 424-5468	Vie
33.	ULRICH, JASON T	15740 N 83RD AVE PEORIA, AZ 85382	24	09/28/1979	(623) 878-4252	Vie
34.	ULRICH, JASON T	8408 W ASTER DR PEORIA, AZ 85381	24	09/28/1979	(623) 878-4252	Vie
35.	ULRICH, JASON T	14840 W ACAPULCO LN SURPRISE, AZ 85379	24	09/28/1979	(623) 878-4252	Vie
36.	ULRICH, JASON T	212 N 44TH ST BELLEVILLE, IL 62226	26	06/20/1978	(618) 939-3983	Vie
37.	ULRICH, JASON T	6970 KONARCIK RD WATERLOO, IL 62298	26	06/20/1978	(618) 939-3983	Vie
38.	ULRICH, JEAN T	RR 1 BOX 76 BELLINGHAM, MN 56212			(320) 568-2313	Vie
39.	ULRICH, JEANNE T	7713 OXON HILL RD OXON HILL, MD 20745	64	01/16/1940		Vie
40.	ULRICH, JEFF T	123 N MAIN ST CHAGRIN FALLS, OH 44022	28	05/1976	(440) 246-1838	Vie
41.	ULRICH, JEFF T	18513 VAN AKEN BLVD SHAKER HEIGHTS, OH 44122	28	05/1976	(216) 246-1838	Vie
42.	ULRICH, JEFF T	411 SW AMBAUM BLVD BURIEN, WA 98166	28	05/1976	(206) 246-1838	Vie
43.	ULRICH, JEFFREY T	18513 VAN AKEN BLVD SHAKER HEIGHTS, OH 44122	28	05/05/1976	(216) 283-7309	Vie
44.	ULRICH, JEFFREY T	7548 CHESTNUT RIDGE RD	28	04/26/1976	(716) 434-8622	Vie

		LOCKPORT, NY 14094				
45.	ULRICH, JEFFREY T	9047 PEARSON RD MIDDLEPORT, NY 14105	28	04/26/1976	(716) 434-8622	Vie
46.	ULRICH, JEFFREY T	913 4TH ST CHARLESTON, IL 61920	30	04/23/1974	(217) 693-7871	Vie
47.	ULRICH, JEFFREY T	THOMAS CHARLESTON, IL 61920	30	04/23/1974		Vie
48.	ULRICH, JEFFREY T	10460 N FOREST TR PEORIA, IL 61615	30	04/23/1974		Vie
49.	ULRICH, JEFFREY T	10460 N FORREST DR PEORIA, IL 61615	30	04/23/1974	(309) 693-7871	Vie
50.	ULRICH, JEFFREY T	5117 W GREENRIDGE CT PEORIA, IL 61615	30	04/23/1974	(309) 693-7871	Vie
51.	ULRICH, JEFFREY T	105 SYCAMORE ST LA CRESCENT, MN 55947	34	10/18/1969	(507) 788-4424	Vie
52.	ULRICH, JEFFREY T	1010 MINNESOTA MINNEAPOLIS, MN 55447	34	10/18/1969	(763) 788-4424	Vie
53.	ULRICH, JEFFREY T	1010 MINNESOTA SAINT PAUL, MN 55114	34	10/18/1969	(612) 788-4424	Vie
54.	ULRICH, JEFFREY T	3174 29TH CT LA CROSSE, WI 54601	34	10/18/1969	(608) 788-4424	Vie
55.	ULRICH, JEFFREY T	939 BETHANNE PL ONALASKA, WI 54650	34	10/18/1969	(608) 788-4424	Vie
56.	ULRICH, JEFFREY T	125 RUSSO DR GUILFORD, CT 6437	37	03/10/1967	(203) 453-8864	Vie
57.	ULRICH, JEFFREY T	30 MOHAWK TRL GUILFORD, CT 6437	37	03/10/1967	(203) 453-8864	Vie
58.	ULRICH, JEFFREY T	30 MOHAWK TRL GUILFORD, CT 6437	37	03/10/1967	(203) 457-1199	Vie
59.	ULRICH, JEFFREY T	522 MORRIS AVE SUMMIT, NJ 7901	37	03/10/1967	(908) 457-1199	Vie
60.	ULRICH, JEFFREY T	280 SAINT JOHNS PL BROOKLYN, NY 11238	37	03/10/1967	(718) 453-8864	Vie
61.	ULRICH, JEFFREY T	280 SAINT JOHNS PL BROOKLYN, NY 11238	37	03/10/1967	(718) 457-1199	Vie
62.	ULRICH, JEFFREY T	33 GOLD ST NEW YORK, NY 10038	37	03/10/1967	(212) 453-8864	Vie
63.	ULRICH, JEFFREY T	33 GOLD ST NEW YORK, NY 10038	37	03/10/1967	(212) 457-1199	Vie
64.	ULRICH, JEFFREY T	280 SAINT JOHNS PL BROOKLYN, NY 11238	37	03/1967	(718) 279-6226	Vie
65.	ULRICH, JEFFREY T	68 CHEEVER PL BROOKLYN, NY 11231	37	03/1967		Vie
66.	ULRICH, JEFFREY T	7231 METROPOLITAN AVE FLUSHING, NY 11379	37	03/1967		Vie
67.	ULRICH, JEFFREY T	1273 3RD AVE NEW YORK, NY 10021	37	03/1967	(212) 279-6226	Vie
68.	ULRICH, JEFFREY T	33 GOLD ST NEW YORK, NY 10038	37	03/1967	(212) 279-6226	Vie
69.	ULRICH, JEFFREY T	1427 EASTWOOD RD	39	11/13/1964	(716) 937-9354	Vie

		ALDEN, NY 14004				
70.	ULRICH, JEFFREY T	46 STUTZMAN RD BOWMANSVILLE, NY 14026	39	11/13/1964	(716) 937-9354	Vie
71.	ULRICH, JEFFREY T	94 ROGERS DR BUFFALO, NY 14225	39	11/13/1964	(716) 937-9354	Vie
72.	ULRICH, JEFFREY T	94 ROGERS DR CHEEKTOWAGA, NY 14225	39	11/13/1964		Vie
73.	ULRICH, JEFFREY T	1427 EASTWOOD RD ALDEN, NY 14004			(585) 937-9354	Vie
74.	ULRICH, JENNIFER T	4578 TRAILS DR SARASOTA, FL 34232	28	05/06/1976		Vie
75.	ULRICH, JILL T	556 S 12 SALT LAKE CITY, UT 84102	38	01/03/1966		Vie
76.	ULRICH, JILL T	575 CREEKVIEW DR SALT LAKE CITY, UT 84107	38	01/03/1966		Vie
77.	ULRICH, JILL T	8101 S 865 SANDY, UT 84094	38	01/03/1966		Vie
78.	ULRICH, JILL T	8402 HALEY CIR SANDY, UT 84094	38	01/03/1966		Vie
79.	ULRICH, JILL T	556 S 12 SLC, UT 84102	38	01/03/1966		Vie
80.	ULRICH, JOAN T	1360 BUNKER HILL DR CHERRY HILL, NJ 8003	62	06/25/1942	(856) 533-1807	Vie
81.	ULRICH, JOAN T	4018 KENSINGTON AVE PHILADELPHIA, PA 19124	62	06/25/1942	(215) 533-1807	Vie
82.	ULRICH, JOHN T	W160N8 MADISON AVE MENOMONEE FALLS, WI 53051	45	11/29/1958		Vie
83.	ULRICH, JOHN T	12820 W CRST GERMANTOWN, WI 53022	46	1958	(414) 242-2272	Vie
84.	ULRICH, JOHN T	15941 W CATSKILL GERMANTOWN, WI 53022	46	1958	(414) 242-2272	Vie
85.	ULRICH, JOHN T	6691 N CHAMPENY SUSSEX, WI 53089	46	1958	(262) 242-2272	Vie
86.	ULRICH, JOHN T	8 LARKSPUR RD LEVITTOWN, PA 19056	51	03/20/1953	(215) 371-5912	Vie
87.	ULRICH, JOHN T	104 WALNUT DR FREDERICKSBURG, VA 22405	51	03/20/1953	(540) 372-3736	Vie
88.	ULRICH, JOHN T	6252 ROCKNOLL LN CINCINNATI, OH 45247	65	08/23/1938	(513) 385-7241	Vie
89.	ULRICH, JOHN T	569 PO BOX CASTLE POINT, NY 12511	66	01/1938	(914) 297-7651	Vie
90.	ULRICH, JOHN T	8 WENDOVER DR POUGHKEEPSIE, NY 12601	66	01/1938	(914) 297-7651	Vie
91.	ULRICH, JOHN T	N113W1 PHEASANT LN GERMANTOWN, WI 53022	69	10/06/1934		Vie
92.	ULRICH, JOHN T	113831 W PHEASANT LN GERMANTOWN, WI 53022	70	1934	(414) 242-3404	Vie
93.	ULRICH, JOHN T	12820 CRESTVIEW DR GERMANTOWN, WI 53022	70	1934	(414) 242-3404	Vie
94.	ULRICH, JOHN T	12820 W CRESTVIEW	70	1934		Vie

		GERMANTOWN, WI 53022				
95.	ULRICH, JOHN T	13831 W PHEASANT LN GERMANTOWN, WI 53022	70	1934	(414) 242-3404	Vie
96.	ULRICH, JOHN T	234 GREENWICH ST BELVIDERE, NJ 7823	78	12/13/1925	(908) 797-0537	Vie
97.	ULRICH, JOHN T	1704 S DAUPHIN ST ALLENTOWN, PA 18103	78	12/13/1925	(610) 797-0537	Vie
98.	ULRICH, JOSEPH T	14 LAKE ST ELLINGTON, CT 6029	54	06/1950	(860) 872-6417	Vie
99.	ULRICH, JOSEPH T	39 WENDELL RD ELLINGTON, CT 6029	54	06/1950	(860) 872-6417	Vie
100.	ULRICH, JOSEPH T	65 SANDY BEACH RD ELLINGTON, CT 6029	54	06/1950	(860) 872-6417	Vie

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Immunology 2004 **111** 306–317

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Early delayed-type hypersensitivity eosinophil infiltrates depend on T helper 2 cytokines and interferon-γ via CXCR3 chemokines

MOE AKAHIRA-AZUMA,* MARIAN SZCZEPANIK,† RYOHEI F. TSUJI,‡ REGIS A. CAMPOS,* ATSUKO ITAKURA,* NARCISS MOBINI,§ JENNIFER MCNIFF,§¶ IVANA KAWIKOVA,* BAO LU,** CRAIG GERARD,** JORDAN S. POBER§¶†† & PHILIP W. ASKENASE*§ **Section of Allergy and Clinical Immunology, Department of Internal Medicine, §Department of Pathology, ¶Department of Dermatology, and ††Section of Immunobiology, Yale University School of Medicine, New Haven, CT, USA, †Department of Human Developmental Biology, College of Medicine, Jagiellonian University, Krakow, Poland, ‡Noda Institute for Scientific Research, Noda, Noda-shi, Chiba-ken, Japan, and **Childrens Hospital, Boston, Harvard Medical School, Boston, MA, USA*

SUMMARY

We investigated the role of T helper (Th)1- and Th2-type cytokines in delayed-type hypersensitivity to soluble protein antigens elicited early postimmunization. Mice were sensitized by intradermal injection without adjuvants, or subcutaneously with complete Freund's adjuvant, and subsequently ear challenged intradermally. As soon as day 3, antigen-specific eosinophil-rich responses were elicited in wild-type mice, but not in T-cell receptor-$\alpha^{-/-}$ mice without adjuvant. Draining lymph node T cells stimulated with antigen secreted interleukin (IL)-4, IL-5 and interferon-γ (IFN-γ). IFN-γ-dependent specific immunoglobulin G (IgG)2a and IL-4-dependent IgG1 were also generated. Delayed-type hypersensitivity ear swelling and local eosinophil recruitment were decreased in IL-$5^{-/-}$, IL-$4^{-/-}$ and signal transducer and activator of transcription-6 (STAT-6)$^{-/-}$ mice, and with anti-IL-4 treatment of wild-type mice, suggesting Th2 mechanisms. Interestingly, responses were also decreased in IFN-$\gamma^{-/-}$ mice, and IFN-γ protein and the IFN-γ-inducible CXC chemokine, IP-10, were present in 24-hr ear tissue extracts, suggesting Th1 effects. Finally, ear swelling, total histology and eosinophils were decreased in mice deficient in CXCR3, the chemokine receptor for IP-10. These results suggest that both a Th2-like (IL-5, IL-4 and STAT-6) and a Th1-like (IFN-γ, IP-10, CXCR3) pathway contribute to eosinophil recruitment in early delayed-type hypersensitivity.

Received 24 July 2003; revised 5 November 2003; accepted 16 December 2003.

Abbreviations: APC, antigen-presenting cell; CFA, complete Freund's adjuvant; CS, contact sensitivity; CXCR3, receptor for IFN-γ-induced CXC chemokines; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; EPO, eosinophil peroxidase; EU, endotoxin units; i.d., intradermal; IFN-γ, interferon-γ; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukin; IP-10, IFN-γ-inducible protein chemokine of 10 000 molecular weight (CXCL10); KLH, keyhole limpet haemocyanin; LNC, lymph node cell; LPS, lipopolysaccharide; mAb, monoclonal antibody; MPO, myeloperoxidase; OVA, ovalbumin; PCl, TNP-chloride (picryl chloride); s.c., subcutaneous; STAT-6, signal transducer and activator of transcription-6; TCR, T-cell receptor; Th, T helper; TNP, trinitrophenyl.

Correspondence: Dr P. W. Askenase, Section of Allergy and Clinical Immunology, Department of Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, PO Box 208013, New Haven, Connecticut 06520-0813, USA. E-mail: philip.askenase@yale.edu

INTRODUCTION

T-cell-mediated immunity *in vivo* mostly proceeds via two different pathways mediated by T helper (Th)1 and Th2-type cytokines. Delayed-type hypersensitivity (DTH) and contact sensitivity (CS) are prototypic skin examples of Th1 reactivity.[1,2] In contrast, Th2 cell-derived interleukin (IL)-4 promotes immunoglobulin E (IgE) and immunoglobulin G1 (IgG1) antibodies that activate mast cells, and induces Th2 effector cells that mediate eosinophil-rich allergic inflammation, as occurs in asthma.[3]

Although immune inflammation often follows these Th1 or Th2 patterns, many natural processes contain cells that produce both types of cytokines, such as in atopic dermatitis,[4,5] rheumatoid arthritis,[6] sometimes in asthma[7–9] and in tumours,[10] and early in intestinal parasite responses.[11] These Th1/Th2 mixtures seem anomalous according to the prevailing paradigm that Th1 and Th2 cells cross-regulate each other via antagonistic cytokines. Thus, we hypothesized some instances of *in vivo* cellular immunity with mixed Th1 and Th2 effects.

Considering DTH as a classical model of *in vivo* T-mediated immunity, we postulated that responses at the onset of elicitability, early postimmunization, might express combined Th1/Th2 cellular immunity. Therefore, in this study we used cytokine knockout mice to study participation of Th1- and Th2-type cytokines in DTH induced at the earliest possible time following a single immunization. Mice were sensitized with a soluble foreign protein antigen that was administered in saline without adjuvants to more closely mimic natural clinical circumstances. We compared early DTH induced by this 'natural immunization' with early DTH following traditional immunization with protein antigen emulsified in mycobacteria-containing complete Freund's adjuvant (CFA), and we also investigated whether mixed Th1 and Th2 effects occur in early elicited CS that is related to DTH.

We found that DTH responses induced without adjuvant and elicited very early postimmunization, contained strong eosinophil infiltrates, accompanied by the *in vitro* production of Th2 (IL-4 and IL-5) and Th1 [interferon-γ (IFN-γ)] cytokines, and possibly by the generation of Th2 (IgG1) and Th1 (IgG2) antibody isotype responses. Classical DTH in mice immunized similarly with CFA also showed strong eosinophil infiltrates when elicited early postimmunization, as did CS responses. Eosinophil recruitment in these early DTH responses was associated with a combination of Th2 cytokines (IL-4, IL-5) and signal transducer and activator of transcription-6 (STAT-6) signalling, and also Th1 cytokines (IFN-γ) that locally generate IFN-γ-induced chemokine IP-10 [IFN-γ-inducible protein chemokine of 10 000 molecular weight (CXCL10)] that acts on CXCR3 receptors. These model responses could relate clinically to diseases in which T-cell-mediated tissue inflammatory responses sometimes also show a mixed Th1/Th2 profile.

MATERIALS AND METHODS

Mice

Six to eight-week-old male CBA/J and female BALB/c/J, C57Bl/6J, C3H/HeN, C3H/HeJ, and immunodeficient T-cell receptor (TCR)$\alpha^{-/-}$ (C57Bl/6), IL-$4^{-/-}$ (BALB/c), STAT-$6^{-/-}$ (BALB/c) and IFN-$\gamma^{-/-}$ (BALB/c) mice (backgrounds in parenthesis) were from Jackson Laboratories (Bar Harbor, ME). Mating pairs of IL-$5^{-/-}$ (B6) mice were from Kim Bottomly (Yale University, New Haven, CT) and IL-$13^{-/-}$ (B6.129) mice were from Andrew McKenzie (Imperial College, London, UK). CXCR3 chemokine receptor-deficient CXCR$3^{-/-}$ (BALB/c) mice were as described previously.[12] Immunodeficient mice were fed autoclaved food and water, housed under pathogen-free conditions using microisolator cages and sterile workbenches, and cared for by gowned and masked personnel. Mice were rested for 1–2 weeks before use, and experiments were performed according to the guidelines of the Animal Care and Use Committee of Yale University School of Medicine.

Reagents

Keyhole limpet haemocyanin (KLH) (Sigma, St Louis, MO; Calbiochem, San Diego, CA) was measured for endotoxin [lipopolysaccharide (LPS)] content by using the Limulus Amebocyte Lysate assay (Associates of Cape Cod Inc., Falmouth, MA). The KLH predominantly used was from Calbiochem (cat no. 374819) and contained significantly less LPS [≈1–19 endotoxin units (EU)/mg of protein] than the KLH from Sigma (catalogue no. H-7017), which contained 2420 EU/mg of protein and was less frequently used. Ovalbumin (OVA) was from Sigma. Anti-mouse IL-4 monoclonal antibody (mAb) (11B11), used at 800 μg/dose, was from Dr Craig Reynolds of the Biologic Branch of the NCI (NIH, Bethesda, MD).

Immunization and skin testing to elicit DTH and CS ear swelling responses

Mice were sensitized by intradermal (i.d.) injection (50 μl × 4) of a total of 100 μg of KLH or OVA in 200 μl of freshly opened sterile pyrogen-free 0·9% saline for injection (Abbot Laboratories No. Chicago, IL), into four divided sites in alcohol-cleaned unshaved abdominal skin. Others received 100 μg of KLH emulsified in CFA (Difco, Detroit, MI) subcutaneously (s.c.) in 50-μl portions into four peripheral sites. In low-responder B6 mice, we used double i.d. (100 μg) immunization in saline on two consecutive days. Littermate controls were injected identically with the saline diluent or CFA alone. For CS, 150 μl of 5% PCl [TNP-chloride (picryl chloride)] in ethanol was applied to the shaved skin and top of the four paws.

On various days after the last sensitization, DTH ear-swelling responses were elicited via bilateral ear i.d. injection under ether anaesthesia with a 10-μl solution containing 5 μg of KLH or OVA in sterile saline (Abbot), or, for CS, by ear paint with 0·4% PCl in olive oil. Controls were similarly ear challenged to obtain background responses. Resulting thickness of the antigen-challenged ears was measured using a dial caliper (Okazaki Mfg., Co., Tokyo, Japan) or a micrometer (Mitutoyo, Kanagawa, Japan), before challenge and at various time-points after challenge. Increased ear thickness was expressed as mean mm $\times 10^{-2} \pm$ SEM. In Fig. 5(a), responses were expressed as net increased ear thickness, by subtracting challenge responses of control unimmunized mice from those of immunized and challenged mice.

Eosinophil peroxidase (EPO) and myeloperoxidase (MPO) activities in DTH ear extracts

Ears were removed 24 hr after challenge and three punch biopsies, 4 mm in diameter, were collected from each ear by using a disposable skin punch (Fray Products, Buffalo, NY).[13] Six biopsies per mouse were extracted using a micro homogenizer (Biospec Products Inc., Racine, WI) in 2 ml of 50 mM potassium phosphate (pH 6·0), containing 0·5% hexadecyl-trimethylammonium bromide, followed by centrifugation at 17 600 *g* for 10 min at room temperature. For MPO activity, extracts were diluted 1 : 10 with 50 mM potassium phosphate (pH 6·0) containing 0·5% hexadecyl-trimethylammonium bromide, and 25 μl was mixed with 225 μl of 50 mM potassium phosphate (pH 6·0) containing 0·167 mg/ml *o*-dianisidine and 0·0005% H_2O_2. For EPO activity, reflecting eosinophil contribution,[13] extracts were diluted 1 : 10 with 50 mM Tris–HCl (pH 8·0) containing 0·1% Triton-X-100, and 50 μl was mixed with 50 mM Tris–HCl (pH 8·0) containing 0·1% Triton-X-100, 1 mM *o*-phenylendiamine and 0·5 mM H_2O_2. After incubation for 30 min at room temperature, the enzymatic reaction was stopped by adding 50 μl of 2 N H_2SO_4 and the absorbance was measured at 450 nm (MPO) or 490 nm (EPO).

In vitro *quantitative measurement of cytokines and chemokines in DTH ear extracts*

After determining the ear thickness at 24 hr, one ear was biopsy punched into three pieces of 4 mm diameter that were frozen immediately in liquid N_2 and stored at $-80°$ until used.[7] Ear pieces were microhomogenized on ice at 100 µl/piece in cold PBS containing proteinase K inhibitor (Boehringer Mannheim, Gmbh, Germany), or 0·05% Tween-20, and then centrifuged at 20 200 *g* for 10 min at 4°. Supernatants were removed and frozen at $-80°$. Quantitative sandwich enzyme-linked immunosorbent assay (ELISA) was performed on ear extracts for IL-4, eotaxin, IL-5, interferon-γ (IFN-γ) and IP-10, using two different specific mAbs [all BD PharMingen, San Diego, CA, except for eotaxin (R & D, Minneapolis, MN) and IP-10 (Leinco Technologies, St Louis, MO)], in 0·1 M $NaHCO_3$ (pH 8–9) at 4°. Wells of microplates were coated overnight with capture mAbs. After blocking with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 25° for 90 min, samples and dilutions of standard recombinant mouse cytokines and chemokines were added (100 µl each) and incubated for 2 hr at 25°. After washing the wells, biotinylated anti-cytokine or anti-chemokine mAbs were added with 1 : 2000 horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA), TMB (tetramethylbenzidine) one-component stop solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used for colour development and the samples were read at 450 nm.

Evaluation of total cell and eosinophil infiltration in DTH

Sections of 5-µm thickness were prepared from formalin-fixed and paraffin-embedded ear reactions and stained with haematoxylin & eosin (H & E) for evaluating the grade of total cell infiltration at low (100×) and high (400×) power. The overall density of inflammation in the dermis was graded by an observer blinded to experimental protocol (N.M. or J.M.), on a scale of 0–4, in a 5-mm length of extravascular dermis along the ear edge starting from the distal ear tip. Grading was as follows: Grade 0, no inflammatory cells; Grade 1, scattered collections of two to five inflammatory cells; Grade 2, scattered infiltrates of ≈20 cells; Grade 3, aggregates of ≥50 inflammatory cells, forming one or more foci; and Grade 4, diffuse infiltration by confluent aggregates of inflammatory cells. Total absolute eosinophil counts were determined in five adjacent 400× fields on sections stained with modified 1% Congo Red (Mallinckrodt, Pittsburgh, PA) in 50% ethanol and then counterstained for 2 min in Mayer's haematoxylin (Shandon, Pittsburgh, PA).[14]

In vitro *quantification of cytokines in supernatants of KLH-stimulated immune lymph node cells (LNC)*

Syngeneic T-cell-depleted single-cell suspensions of splenocytes were used as antigen-presenting cells (APC) and prepared by incubation with mAb to CD4 (GK1.5), CD8 (TIB 105) and Thy-1 (Y19) (gift of Kim Bottomly), followed by treatment at 37° for 45 min with mitomycin C (5 µg/ml) and a previously determined dilution of rabbit serum complement (Pelfreeze, Brown Deere, WI). Single-cell suspensions of axial and inguinal LNC from KLH-immunized mice were prepared, and 4×10^6 LNC/ml were cultured with 2×10^6 APC/ml, in 2-ml wells, with KLH (100 µg/ml) in RPMI-1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum, at 37° for 48 hr, in flat-bottom 24-well microplates (Falcon, Oxford, CA). Supernatants were collected at 48 hr for assay of cytokine production using commercial ELISA kits (BD PharMingen).

Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Fisher's test and, in some instances, by the Student's *t*-test. A difference was considered statistically significant at a *P*-value of $<0·05$.

RESULTS

Time course and antigen specificity of DTH ear-swelling responses after i.d. immunization with KLH, and the role of LPS

Following KLH immunization in saline alone, DTH ear swelling was elicited in CBA/J mice on days 3 and 5 (Fig. 1a) and persisted at similar levels for 96 hr (Fig. 1a). DTH was first elicited on day 3 and persisted for at least 21 days (Fig. 1b). We considered that LPS contamination of KLH might be involved in early DTH responsiveness. However, this was not the case because KLH preparations with widely varying levels of LPS (1–2400 EU/mg) induced similar DTH (data not shown), and immunization of LPS-unresponsive C3H/HeJ mice produced DTH reactions similar to those of LPS-responsive C3H/HeN mice (Fig. 1c). To investigate antigen specificity we compared 24-hr ear swelling induced by i.d. immunization with OVA in saline. KLH challenge of KLH-immunized mice elicited DTH (Fig. 1d, Group B), but challenge with OVA did not (Group C). In contrast, OVA-induced DTH was elicited with OVA (Group E), but not with KLH (Group F). These results indicated that early DTH was antigen-specific.

Analysis of infiltrating inflammatory cells and leucocyte enzymes

Large numbers of eosinophils were found in infiltrates, which showed an increase at 18 hr, peaked at 24 hr and remained elevated to the 48-hr time-point; these eosinophils comprised 30–90% (average 60%) of the total infiltrate (Fig. 2a). The presence of eosinophils was confirmed by electron microscopy showing cytoplasmic granules with distinctive crystalline cores (Fig. 2b, and inset) and by biochemical assay of eosinophil-derived EPO (Fig. 2c) versus neutrophil-derived MPO (Fig. 2c) in 24-hr responses versus controls (Group B versus A).

Th1 and Th2 pattern of induced cytokines and serum antibodies

By day 5 postimmunization, an *in vitro* culture of mixed LNC and APC produced IL-4 (±200 pg/ml), IL-5 (±200 pg/ml) and IFN-γ (13 ng/ml) when stimulated with 100 µg/ml KLH, compared to stimulation with no antigen. Titres of specific anti-KLH IgG isotypes by ELISA showed no specific IgG antibodies on days 3 or 5, but, by day 21, anti-KLH IgG1, IgG2a and IgG2b

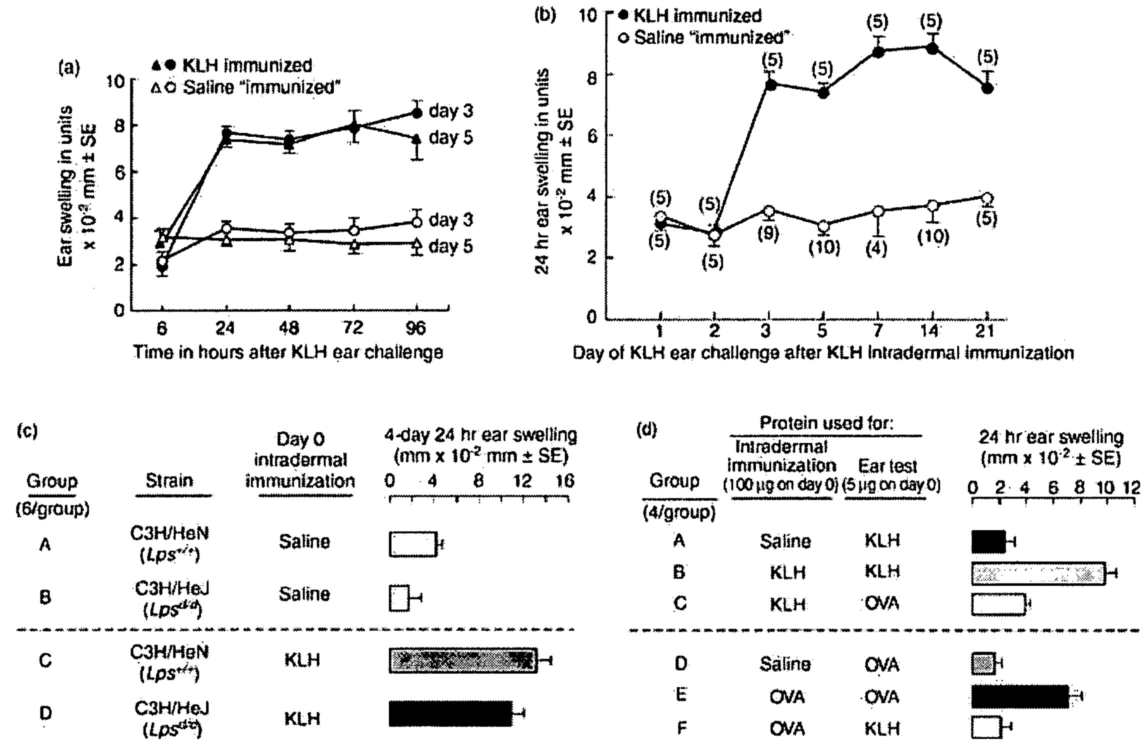


Figure 1. Keyhole limpet haemocyanin (KLH) intradermal (i.d.) immunization induces 24–96-hr antigen-specific delayed-type hypersensitivity (DTH) elicitable on day 3 and day 5. (a) Time course of KLH-elicited DTH ear swelling in CBA/J mice on day 3 or on day 5 post i.d. immunization in saline. At the indicated time-points after challenge, ear swelling was determined using a micrometer, subtracting elicited ear thickness at each time-point from the thickness prior to challenge. The open circles and triangles represent saline-'immunized' and KLH ear-tested controls on day 3 and day 5, respectively. The closed circles and triangles represent KLH-immunized and KLH ear-tested experimental mice on day 3 and day 5, respectively. (b) 24-hr DTH ear swelling on various days postimmunization of CBA/J male mice. The open circles represent saline-immunized controls and the closed circles represent KLH-immunized mice; both ear challenged with KLH. Numbers in parenthesis represent the total numbers of mice per time-point. (c) Induction of KLH DTH in lipopolysaccharide (LPS)-unresponsive C3H/HeJ (Group D) versus LPS-responsive C3H/HeN mice (Group C) compared with saline-'immunized' controls (Groups A and B), tested on day 4. There was no difference in elicited 24-hr DTH. (d) To test for the antigen specificity of 24-hr DTH, CBA/J mice were immunized i.d. (without adjuvants) on day 0 with 100 μg of KLH or ovalbumin (OVA) in saline. On day 4, each type of immunized mice was challenged i.d. in the ears by injection of 5 μg of KLH or OVA. Twenty-four hours following challenge, ear thickness was determined to compare antigen specificity of KLH versus OVA-induced DTH (Group B versus A and Group B versus C; $P < 0.001$; Group E versus F, and Group E versus D; $P < 0.002$).

were detected in serum (data not shown). Therefore, the *in vivo* generation of specific anti-KLH IgG isotypes and KLH-induced cytokines in immune LNC *in vitro* were consistent with the idea that DTH responses might depend on a mixture of Th1- and Th2-type cytokines.

TCR-$\alpha^{-/-}$, IL-$5^{-/-}$, IL-$4^{-/-}$ and STAT-$6^{-/-}$ mice have decreased DTH and eosinophils

On days 3 and 5 in TCR-$\alpha^{-/-}$ mice, macroscopic DTH (Fig. 3; 24-hr ear swelling column) total cells and eosinophils were not elicited (Groups D and F), compared with wild-type controls (Groups C and E). Treatment of adoptive-transferred cells with anti-CD4 mAb plus complement abrogated the DTH elicited in recipients (data not shown), suggesting mediation by CD4$^+$ TCR-$\alpha\beta^+$ Th cells. The dominant eosinophils suggested involvement of Th2 cytokines. Tests in IL-$5^{-/-}$ mice showed that the 24-hr ear swelling was significantly decreased (Fig. 4, ear swelling column, Groups D and F) compared with wild-type controls (Group C and E), but was not fully inhibited, while total cell infiltration (Fig. 4), and especially infiltrating ear eosinophils (Fig. 4) were strongly decreased (Group C versus D and Group E versus F). These results suggest that IL-5 contributed to macroscopic and eosinophil aspects of early DTH. Macroscopic DTH was absent in IL-$4^{-/-}$ BALB/c mice on day 3, was impaired significantly on day 4, but was indistinguishable from controls by days 5 and 6 (Fig. 5a). Eosinophils also were decreased significantly in IL-$4^{-/-}$ versus IL-$4^{+/+}$ mice on day 3 and 5 (Fig. 5b, Group C versus D), and still showed a marked decreased on day 5 (Group E versus F). Together these results indicated that both IL-4 and IL-5 contribute to early KLH DTH and eosinophil infiltrates but, by day 5, IL-4 and IL-5 contribute weakly to macroscopic swelling. As IL-4 deficiency was present before sensitization and challenge, it was impossible to determine which aspect of DTH was IL-4 dependent. Thus, we performed experiments with neutralizing anti-IL-4 mAb given at various time-points in

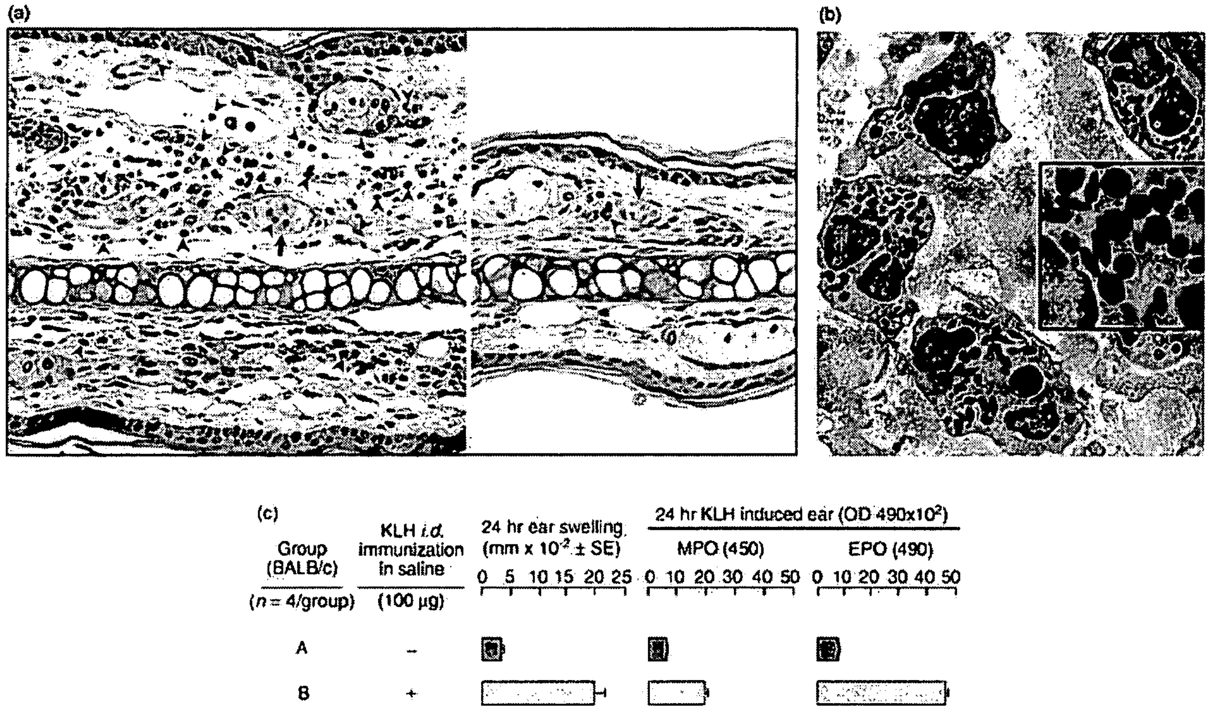


Figure 2. Keyhole limpet haemocyanin (KLH) induced delayed-type hypersensitivity (DTH): light (a) and electron (b) microscopy, and local myeloperoxidase (MPO) and eosinophil peroxidase (EPO) levels (c). (a) Light microscopic histology (100× magnification) of the 24-hr KLH DTH response elicited on day 5 post intradermal (i.d.) immunization with KLH in abdominal skin in a wild-type mouse (left panel), compared with a non-immune mouse (right panel), both challenged in the ears with 5 μg of KLH in saline. The horizontally aligned aerated cartilage in the center of the ear sections allows comparison, showing that the immune mouse ear (left) is quite swollen, and contains a strong, mostly dermal, cellular infiltrate of dominant eosinophils, many with evident bilobed nuclei and Congo Red-stained ctyoplasmic granules (indicated by arrowheads). Also, a dilated microvessel was shown to contain eosinophils (indicated by an arrow). The panel on the right shows a similar 24-hr ear section from a non-immune and similarly KLH ear-challenged mouse, with little ear swelling, few inflammatory cells, a rare eosinophil (indicated by an arrowhead) and a similar microvessel without eosinophils (indicated by an arrow). (b) Low-power electron micrograph of an ear piece sectioned from near the center of a KLH-elicited 4-day DTH ear response. Tissue was fixed in paraformaldehyde/glutaraldehyde, embedded in LX-112 resin, and cut into 800-Å sections, and showed dermal accumulation of many eosinophils (four are shown at a 15 500× magnification), with distinctive granules containing crystalline cores (inset, 31 500× magnification). (c) MPO that is found predominately in neutrophils (middle), and EPO that is found predominately in eosinophils (right), were assayed in extracts of ears excised from 24-hr DTH responses (left) of CBA/J mice immunized with KLH in saline and ear tested on day 4 (Group B), compared with saline-immunized controls (Group A).

CBA/J mice, to also explore the role of IL-4 in another strain. Treatment with anti-IL-4 before immunization and before elicitation, significantly inhibited DTH (Fig. 5c, Group C versus B), confirming IL-4 involvement. Furthermore, mice that received anti-IL-4 just prior to immunization also had decreased DTH (Fig. 5c, Group D), suggesting that IL-4 may have a role in the induction of DTH. However, anti-IL-4 IgG, with a half-life of ≈20 days, may have been present at the time of DTH elicitation on day 4 and therefore may also have affected DTH elicitation. In fact, anti-IL-4, given on day 3 alone, just 1 day prior to elicitation, was also inhibitory (Fig. 6c, Group E), suggesting that IL-4 may be involved in the efferent phase. Similar KLH-immunized and challenged mice that received irrelevant mAb of the same isotype, both before immunization and testing, had no impaired DTH (Group F). In contrast, mice deficient in IL-13, that can also recruit eosinophils and utilize the STAT-6 pathway, showed no decrease of DTH (Fig. 5d) or eosinophils (data not shown). As STAT-6 is a crucial signalling molecule for receptors stimulated by IL-4, we tested the effect of STAT-6 deficiency. Macroscopic 24-hr-elicited DTH on day 3 was moderately, but significantly, decreased (Fig. 6a, 24-hr ear swelling column, Group C versus D), but by day 5 there was no decrease (Group E versus F). In contrast, eosinophil infiltrates were inhibited strongly and significantly (Fig. 6a, ear eosinophils column, Group D and F), confirming findings in IL-4$^{-/-}$ and IL-5$^{-/-}$ mice. By day 5, stronger macroscopic DTH was generally elicited and was less dependent on Th2 cytokines, while eosinophil infiltrates remained dependent on IL-5 (Fig. 4, ear eosinophils column), IL-4 (Fig. 5b) and STAT-6 signalling (Fig. 6a, ear eosinophils column), suggesting that a non-Th2 process might also be involved.

IFN-γ dependence of early elicited DTH

As Th2-type cytokines did not entirely account for the early DTH response, we investigated whether IFN-γ, the prototype Th1 cytokine, was also involved. Interestingly, positive 24-hr macroscopic DTH was markedly decreased in IFN-γ$^{-/-}$ mice on both day 3 (Fig. 7, ear swelling, Group C versus D) and on day 5

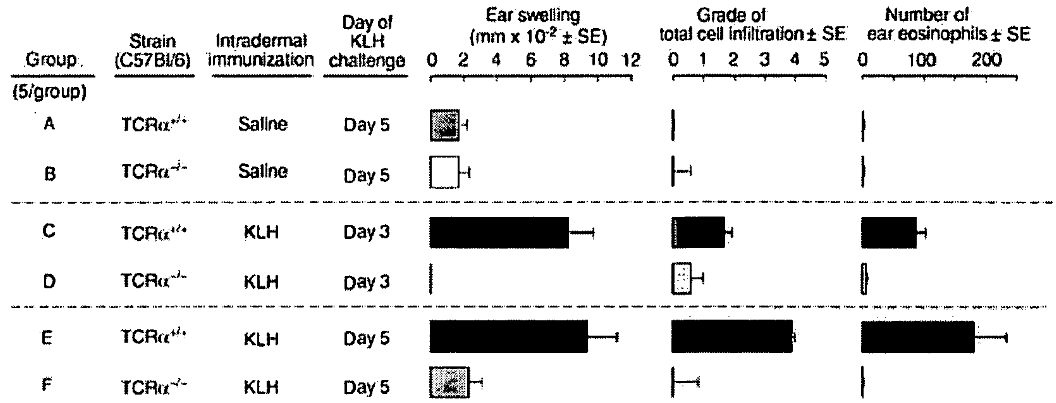


Figure 3. Keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) is mediated by T-cell receptor (TCR)-$\alpha\beta^+$ T cells. TCR-$\alpha^{-/-}$ and control C57Bl/6 mice received double intradermal (i.d.) immunization with 100 μg of KLH on two consecutive days, and then were ear challenged i.d. with 5 μg of KLH on day 3 or 5 after immunization. Ear thickness at time 0 versus 24 hr (left) and 24 hr total cell infiltration (middle) and eosinophil counts (right) were measured and found to be totally dependent on αβ T cells (Group C versus D, and E versus F, $P < 0{\cdot}001$, for ear swelling, total infiltrates and number of eosinophils).

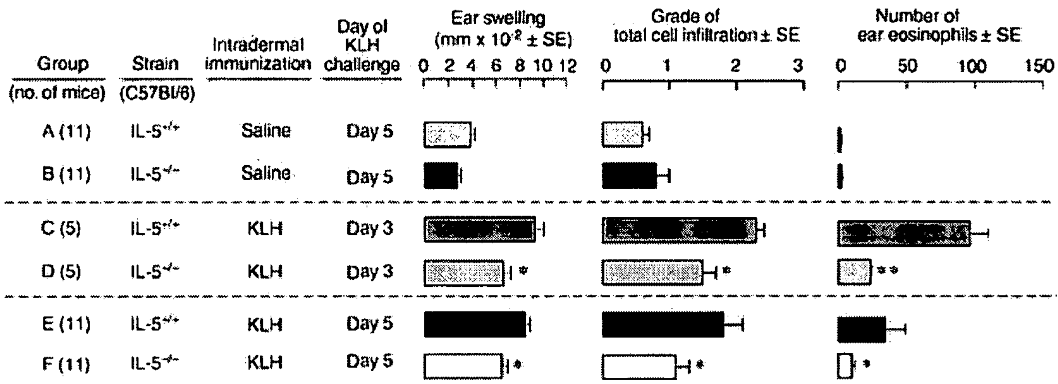


Figure 4. Twenty-four hour keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) ear swelling (left), grade of total cell infiltrate (middle) and eosinophils (right) in interleukin (IL)-$5^{-/-}$ compared with IL-$5^{+/+}$ mice. Left panel: IL-$5^{-/-}$ and control B6 mice immunized with 100 μg of KLH, ear challenged and DTH responses determined by ear thickness (*Group C versus D, $P < 0{\cdot}01$; *Group E versus F, $P < 0{\cdot}01$). Middle panel: cellular infiltration determined along 5 mm of the ear edge starting from the tip. On days 3 and 5, cell infiltration was found to have increased in IL-$5^{+/+}$ mice (Groups C and E) and decreased significantly on days 3 and 5 in IL-$5^{-/-}$ mice (Group D and F) (*Group C versus D, $P < 0{\cdot}03$; *Group E versus F, $P < 0{\cdot}04$). Right panel: total eosinophils were increased in KLH DTH in IL-$5^{+/+}$ mice (Groups C and E), compared with Group A saline-immunized mice, while eosinophils were decreased significantly in KLH-immunized and challenged IL-$5^{-/-}$ mice (Groups D and F). (**Group C versus D, $P < 0{\cdot}001$; *Group E versus F, $P < 0{\cdot}04$).

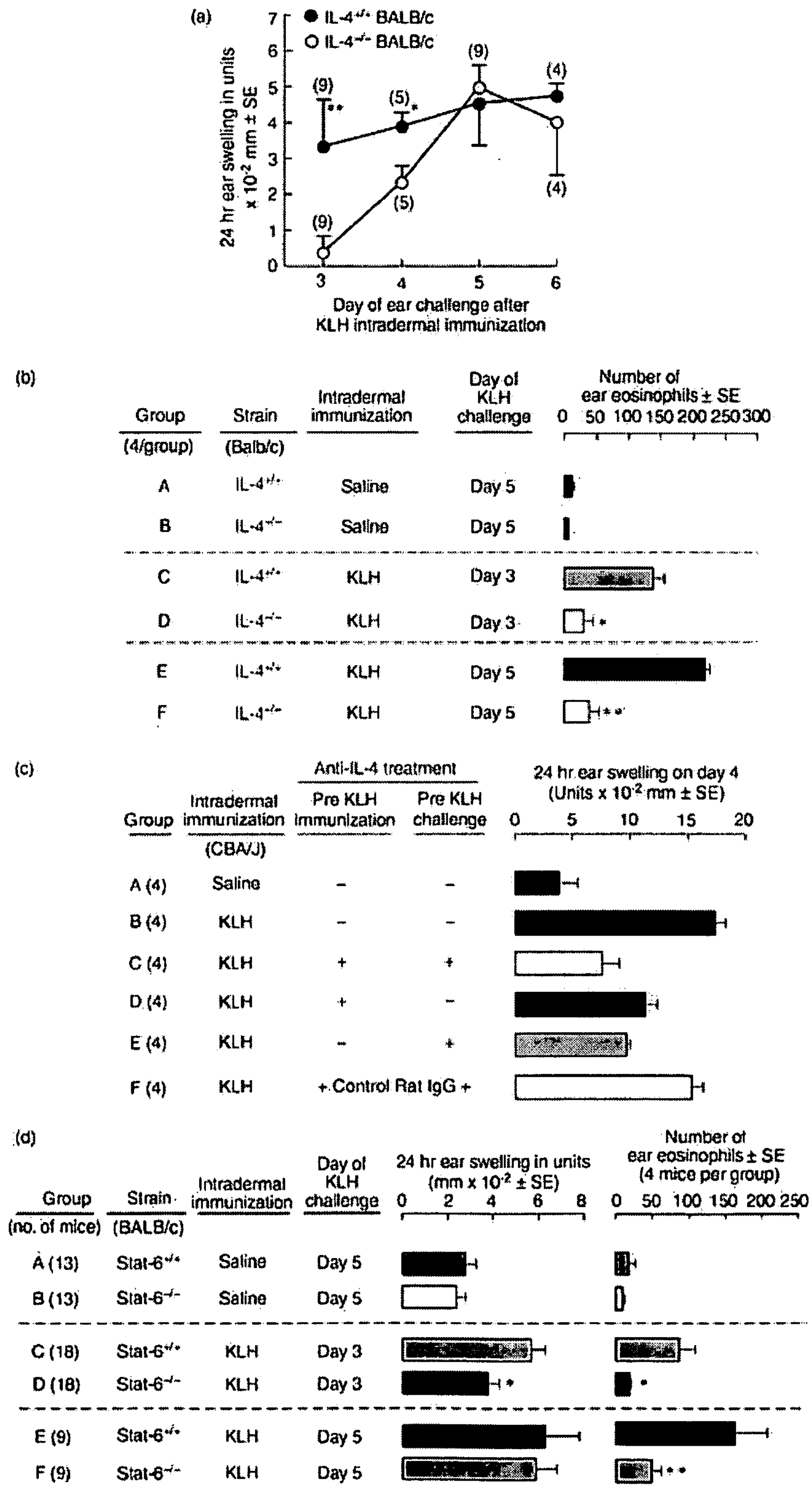
(Group E versus F). Total cellular infiltration appeared to be less dependent upon IFN-γ, but surprisingly, the number of eosinophils, especially on day 5, was also reduced markedly in IFN-$\gamma^{-/-}$ mice (Fig. 7, ear eosinophils, Group E versus F), suggesting involvement of Th1-derived IFN-γ. The lack of change in total cells in the IFN-$\gamma^{-/-}$ mice that had depleted eosinophils on day 3, was the result of a compensatory increase in the number of mononuclear cells.

Eosinophil-rich early DTH in mice immunized with CFA, and in CS

We considered that the finding of eosinophil-rich DTH could have been the result of elicitation of responses very early postimmunization and not because of the lack of adjuvant use. Thus, CBA/J mice were similarly immunized, but this time s.c. with 100 μg of KLH in saline emulsified with CFA; the results were compared to i.d. immunization with saline, and mice were identically ear tested with KLH on day 3 or day 5. No response was elicited on day 3 (data not shown), but DTH was elicited on day 5 and, compared to immunization with KLH plus saline but without CFA, was somewhat macroscopically stronger (Fig. 8a, Group B versus D, first column), had a greater number of total infiltrating cells (second column) and, remarkably, had a similar number of eosinophils (third column), with an accompanying increased correlating EPO (fourth column). We concluded that eosinophil-rich DTH responses were elicited early postimmunization, in mice immunized with protein administered either with or without adjuvant (CFA).

Early eosinophils in CS a form of DTH induced without adjuvants by skin painting with reactive hapten chemicals

CS induced by concentrated PCl skin painting was elicited on day 4 by ear challenge with dilute PCl, and the resulting 24-hr ear-swelling responses also contained significant eosinophil infiltrates (Fig. 8b). Thus, a high level of eosinophil infiltrates was a property of various types of DTH elicited early postimmunization.



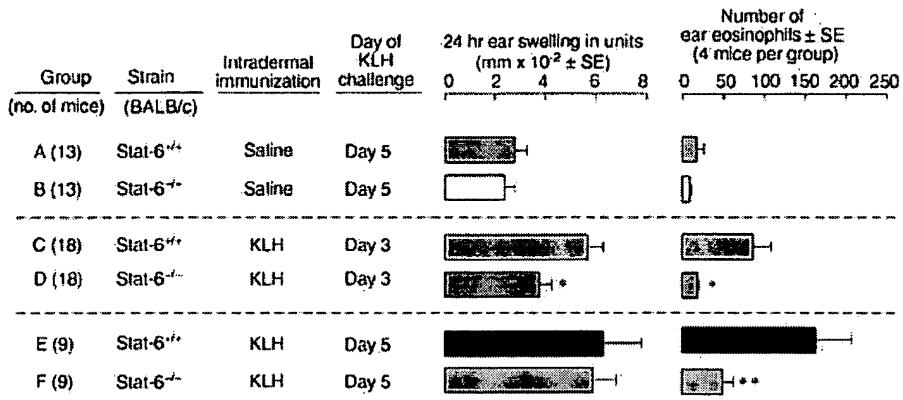


Figure 6. Macroscopic keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) and eosinophil infiltrates are decreased in STAT-$6^{-/-}$ mice. Left column: macroscopic 24-hr KLH DTH was elicited on day 3 or 5 in STAT-$6^{+/+}$ mice, but was decreased significantly on day 3, but not on day 5, after immunization in STAT-$6^{-/-}$ mice (*Group C versus D, $P < 0{\cdot}03$). Right column: 24-hr ear eosinophil infiltration was increased on day 3 and day 5 after KLH immunization in STAT-$6^{+/+}$ mice (Groups C and E) and was decreased significantly on day 3 and day 5 in STAT-$6^{-/-}$ mice (Group D and F) (*Group C versus D, $P < 0{\cdot}05$; **Group E versus F, $P < 0{\cdot}002$).

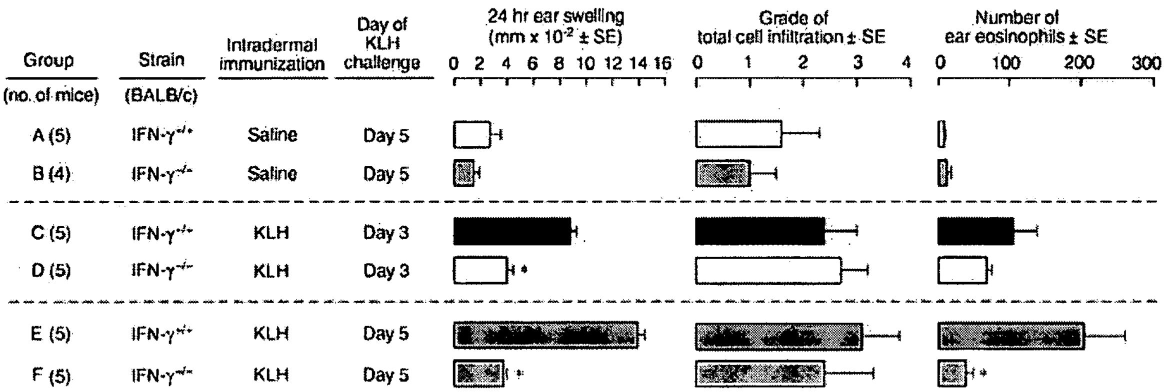


Figure 7. Keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) is dependent on interferon-γ (IFN-γ) on day 3 and day 5 after immunization. Left column: DTH was elicited on day 3 and 5 in IFN-$\gamma^{+/+}$ mice (Group C and E), but was decreased significantly on day 3 and 5 after immunization of IFN-$\gamma^{-/-}$ mice (Group D and F) (*Group C versus D, $P < 0{\cdot}01$; *Group E versus F, $P < 0{\cdot}001$). Middle column: the total grade of cell infiltration was increased on day 3 and day 5 in IFN-$\gamma^{+/+}$ mice (Group C and E), and was not decreased on day 3 or 5 in IFN-$\gamma^{-/-}$ mice. Right column: eosinophil infiltration in DTH ears was increased on day 3 and 5 after KLH in wild-type IFN-$\gamma^{+/+}$ mice (Group C and E, versus Group A), and was decreased significantly in IFN-$\gamma^{-/-}$ mice on day 5 (*Group E and F, $P < 0{\cdot}001$). However, although decreased on day 3, this was not significant ($P < 0{\cdot}1$).

Local production of cytokines and chemokines in DTH and CS ear responses

IFN-γ-dependent eosinophil recruitment in DTH induced without adjuvant was unusual (Fig. 7), as were similar eosinophil-rich infiltrates and local EPO activity induced with CFA immunization (Fig. 8a), that instead was expected to induce Th1 cytokines mediating eosinophil-poor responses. Furthermore, finding eosinophil-rich early CS responses indicated that this finding was widespread. We considered that IFN-γ released by

Figure 5. Interleukin (IL)-4 is involved in keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) elicited early after immunization on days 3 and 4, but not on days 5 and 6; IL-13 is not involved. (a) KLH DTH was elicited in wild-type IL-$4^{+/+}$ mice on days 3–6 and was strongly decreased on day 3 in IL-$4^{-/-}$ mice, less so on day 4, and then not at all on days 5 or 6 (**day 3, $P < 0{\cdot}005$; *day 4, $P < 0{\cdot}01$). (b) Eosinophil infiltration was found to be increased in IL-$4^{+/+}$ mice on days 3 and 5 after KLH immunization and showed a significant decrease in IL-$4^{-/-}$ mice (*Group C versus D, $P < 0{\cdot}05$; **Group E versus F, $P < 0{\cdot}003$). (c) Anti-IL-4 or control rat immunoglobulin G (IgG) was given intraperitoneally (i.p.), either 2 hr before immunization or 1 day before challenge, or at both time-points in separate groups of CBA/J mice. DTH responses on day 4 were inhibited by anti-IL-4 treatment, both in mice treated prior to immunization (Group D) and prior to ear challenge (Group E), and in mice treated at both time-points (Group C), but not in controls treated similarly with rat IgG (Group F) (Group B versus C, $P < 0{\cdot}001$; Group B versus D, $P < 0{\cdot}004$; Group B versus E, $P < 0{\cdot}002$). (d) Elicitation of 24-hr KLH DTH was attempted on days 3 and 5 after immunization in IL-$13^{-/-}$ and in control IL-$13^{+/+}$ mice, and was found to be identically positive in both strains on both days.

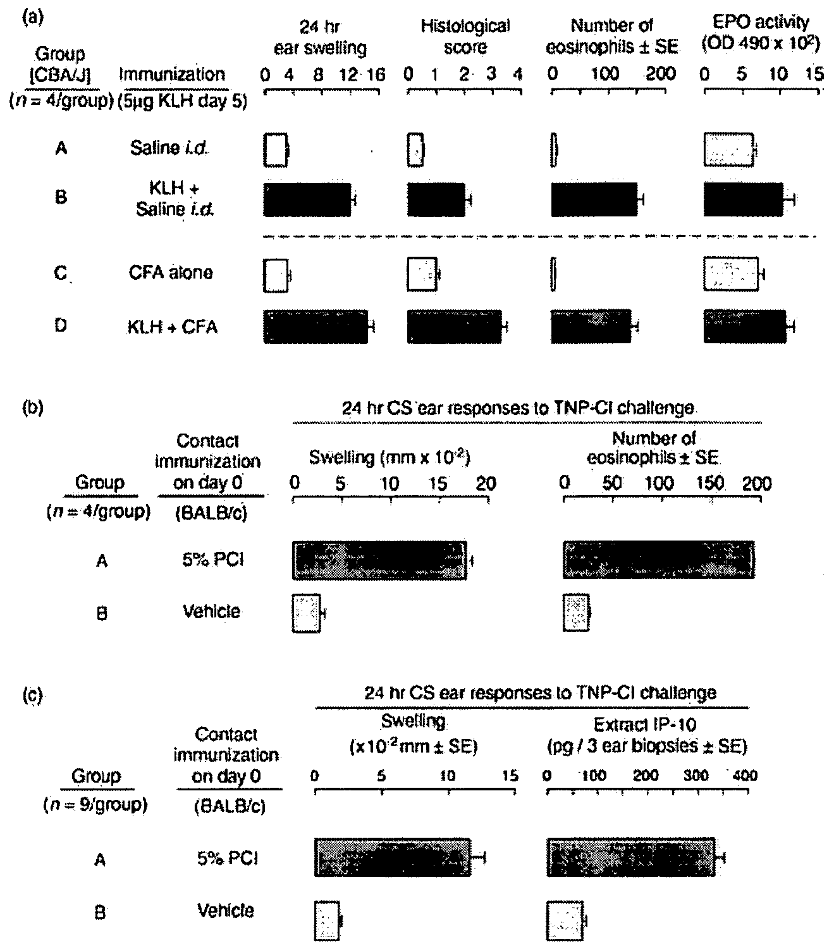


Figure 8. (a) Comparison of delayed-type hypersensitivity (DTH) induced by keyhole limpet haemocyanin (KLH) administered intradermally (i.d.) in saline, versus DTH induced by KLH emulsified in complete Freund's adjuvant (CFA) and administered subcutaneously (s.c.). Mice were immunized with KLH i.d. in saline without adjuvant (Group B), and DTH elicited on day 5 was compared to responses on day 5 of mice immunized s.c. with KLH emulsified in mycobacterial CFA adjuvant (Group D). Both immunizations induced a DTH that was similar in macroscopic swelling (first column), total cell infiltrate (second column), total eosinophils (third column) and eosinophil peroxidase (EPO) activity (fourth column), compared to controls immunized with saline (Group A) or CFA alone (Group C). The differences in all columns, between experimental Groups B and D, were not significant. (b) Early elicited eosinophil-rich 24-hr reaction in BALB/c mice immunized for contact sensitivity (CS). Female, 6–7-week-old BALB/c mice were contact sensitized by painting the abdomen, chest and hind feet with 5% PCl [TNP-chloride (picryl chloride)] and ear challenged with 0·4% PCl on day 4. Ear swelling and local eosinophil counts were significantly increased in actively sensitized and challenged mice (Group A) compared to vehicle-immunized and similarly challenged controls (Group B) ($P < 0{\cdot}001$, Group A versus B, left column; $P < 0{\cdot}001$, Group A versus B, right column). (c) Interferon-γ (IFN-γ)-induced IP-10 (CXCL10) chemokine in the PCl contact sensitivity of BALB/c mice. Female BALB/c mice were contact sensitized with 5% PCl and ear challenged with 0·4% PCl on day 4. Ear swelling and the accompanying ear extract content of IP-10 were significantly increased in sensitized and challenged mice (Group A) compared to similarly immune and challenged control mice ($P < 0{\cdot}001$, Group A versus B, 24-hr CS $P < 0{\cdot}001$, Group A versus IP-10 control).

Th1 DTH effector T cells might have induced local tissue cells to produce chemokines that led to the eosinophil accumulation, as IFN-γ-induced CXC chemokines such as IP-10 (CXCL10)[15] can activate CXCR3 receptors expressed on activated human eosinophils[16] or on Th0 and Th1 cells.[17,18]

To test for this unusual pathway, we quantified IFN-γ and IP-10 polypeptides in 24-hr DTH ear extracts. Figure 9(a) shows elevated IFN-γ levels in 24-hr ear extracts compared with controls (left column, Groups B and C versus A), and increased IP-10 in mice similarly immunized without adjuvant (right column, Groups B and C, versus A). Furthermore, IFN-γ and IP-10 were expressed significantly in DTH induced with KLH in CFA (data not shown), that similarly was eosinophil-rich (Fig. 8a, third column) compared to controls immunized with CFA alone. Finally, IP-10 was also elevated in PCl-induced CS (Fig. 8c), indicating that IP-10-associated eosinophil infiltrates were characteristic of the three varieties of early elicited DTH, i.e. induced with and without CFA adjuvant, and in early CS. We attempted to evaluate the number of Th2 cytokines and related chemokines in ear extracts, but eotaxin background levels in normal ears were too high to permit analysis by standard ELISA. Furthermore, neither IL-4 nor IL-5 could be detected

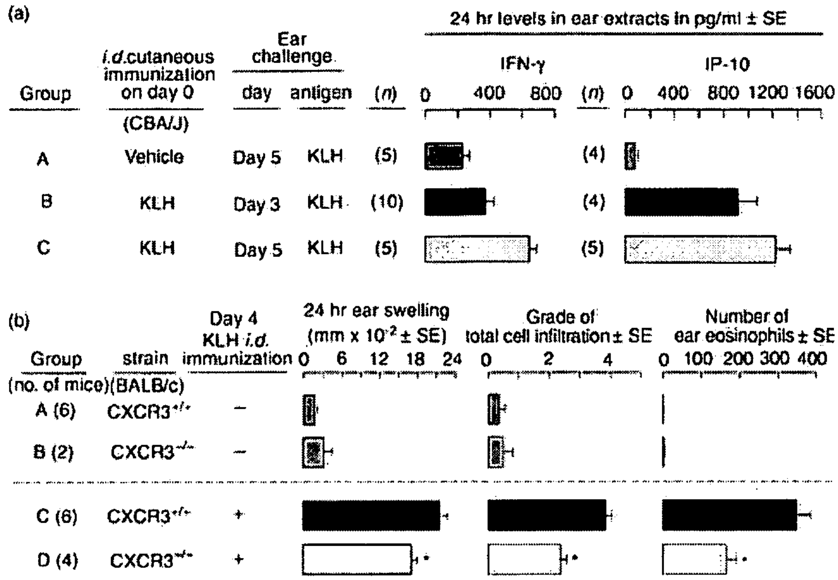


Figure 9. Production of interferon-γ (IFN-γ) and IP-10 [IFN-γ-inducible protein chemokine of 10 000 molecular weight (CXCL10)] in 24-hr keyhole limpet haemocyanin (KLH) delayed-type hypersensitivity (DTH) ear extracts, and dependency of eosinophilic DTH on CXCR3 chemokine receptors. (a) At 24 hr after intradermal (i.d.) KLH ear challenge of KLH-immunized CBA/J mice, extracts of ear-swelling responses of mice tested on days 3 and 5 were assayed for IFN-γ content (left) and IP-10 content (right) by quantitative enzyme-linked immunosorbent assay (ELISA). IFN-γ and IP-10 were found in extracts of day-5 DTH responses (Group C). A less significant increase in IFN-γ, but equivalent IP-10, were found on day 3 (Group B), when compared to similarly KLH-challenged 24-hr ear extracts from vehicle-immunized controls (Group A) (Group A versus C for IFN-γ and IP-10, $P < 0{\cdot}0001$; Group A versus B for IFN-γ, $P < 0{\cdot}01$; Group B versus C for IFN-γ and IP-10, $P < 0{\cdot}05$). (b) Macroscopic DTH, total cellular infiltrate and eosinophil accumulations are decreased in 24-hr KLH DTH responses of CXCR3$^{-/-}$ mice (BALB/c), compared with wild-type controls. Left column: decreased 24-hr macroscopic KLH DTH was elicited on day 4 in immunized CXCR3$^{-/-}$ compared with wild-type mice. Middle column: total cellular infiltration was decreased in DTH responses of KLH-immune CXCR3$^{-/-}$ mice compared with KLH-immune wild-type (BALB/c) mice. Right coumn: eosinophil accumulations were decreased in DTH responses of KLH-immune CXCR3$^{-/-}$ mice compared with wild-type controls (*$P < 0{\cdot}05$, Group D versus C for left, middle and right columns).

in ear extracts, although, as mentioned previously, the same ELISA showed that these cytokines were stimulated *in vitro* in day 5-harvested KLH-immune LNCs. Thus, IL-4 and IL-5 were probably produced by DTH-immune T cells, or other cells present, but the quantity produced locally was probably below the lower limit of detection by ELISA.

DTH in CXCR3-deficient mice

As 24-h KLH DTH ear extracts showed local elaboration of IFN-γ and IP-10, and because IP-10 solely interacts functionally with CXCR3 chemokine receptors, we evaluated DTH in CXCR3 chemokine receptor-deficient mice.[19] We found a small, but significant, decrease in macroscopic DTH in CXCR3$^{-/-}$ versus control BALB/c mice (Fig. 9b, ear swelling, Group C versus D). Furthermore, there was a decreased total cell infiltrate (Group C versus D) and, in particular, a significantly decreased number of ear eosinophils (Fig. 9b, Group C versus D). These results correlated with the decreased DTH and eosinophils in IFN-γ$^{-/-}$ mice (Fig. 7), and with the presence of both IFN-γ and IP-10 in extracts of DTH from wild-type mice induced without (Fig. 9a) or with (data not shown) adjuvant, or in PCl-induced CS (Fig. 8b,c). Thus, these results suggest a Th1-type pathway of eosinophil infiltration in DTH that is dependent on IFN-γ, IP-10 and CXCR3.

DISCUSSION

DTH responses studied at their onset, on days 3–5 postimmunization, showed that a Th1 and Th2 mixed response was elicited. Early elicited DTH induced without adjuvant depended on a combination of Th1 (IFN-γ) and Th2 (IL-4, IL-5 and STAT-6 signalling) cytokines and was rich in eosinophils. Furthermore, the presence of eosinophils, IFN-γ and IP-10 in DTH responses induced with CFA, and in CS, led to the postulate that a Th1/Th2 mixed-effector reaction may be mediated by Th0 cells early postimmunization. Involvement of IFN-γ in eosinophil-rich DTH probably depended on the local induction of IP-10 to trigger CXCR3, possibly on T cells.[17,18] Importantly, early elicited combined Th/Th2 DTH was present in all strains tested, including CBA/J, C3H, C57Bl/6 and BALB/c, and thus was not a property of a particular genetic background.

Special properties of early elicited DTH

By performing the study from the very start of DTH elicitability, we optimized the chances that DTH was examined in relative isolation, without later coincident inflammatory responses to antigen, possibly as a result of IgG or IgE antibodies activating mast cells, or IgG isotypes activating complement. Taken together, our results overturn several rules about DTH:

(1) Antigen-specific responses can be elicited without adjuvant quite early postimmunization.
(2) Eosinophil-rich DTH was elicited in several different background strains and was not reduced by the addition of a strongly Th1-inducing CFA adjuvant, and also characterized early elicited CS.
(3) Most surprisingly, the prototypic Th1 cytokine, IFN-γ, is involved in generating the eosinophils, probably by inducing the chemokine IP-10 to activate CXCR3.

Eosinophil recruitment in DTH

Mechanisms for eosinophil recruitment into immune inflammation mostly involve the Th2 cytokine and chemokine pathway. Early eosinophil-rich DTH fits this scenario, in part, because IL-5- and IL-4-deficient mice showed impaired macroscopic DTH and decreased eosinophil infiltrates, as did STAT-$6^{-/-}$ mice. However, we have not definitively shown that Th2 cytokines are locally produced by T cells, and certainly other cells may also be involved. Importantly, responses were not eliminated in mice deficient in Th2 cytokines and IFN-γ also contributed. These findings were extended by the use of Th1 promoting CFA, that when used as adjuvant also led to large eosinophil infiltrates in early DTH. Eosinophil-rich responses also occurred in early elicited CS. An alternative IFN-γ-mediated Th1 pathway of eosinophil-rich tissue responses is not unprecedented. In tuberculosis, some patients have pleuritic inflammation rich in eosinophils[19,20] and eosinophilic pleurisy is induced in mice by intrapleural injection of mycobacteria.[21–23] Importantly, IFN-γ-dependent recruitment of activated eosinophils into CFA-induced Th1-dominant DTH was demonstrated recently.[24] CXC chemokines (such as IP-10) induced by IFN-γ, and that bind to corresponding CXCR3 receptors, may be involved in early DTH that is rich in eosinophils. DTH induced with and without CFA, and also PCl-induced CS, showed elevated IP-10 in 24-hr ear extracts, and mice deficient in CXCR3 had significantly reduced macroscopic, total infiltrate and eosinophil aspects of KLH DTH. These findings strongly suggest that IFN-γ-induced IP-10 interacting with CXCR3 is involved in DTH and CS. However, it is not clear which cells express the involved CXCR3. Although CXCR3 have been described on human eosinophils,[16] we were unable to demonstrate CXCR3 on mouse eosinophils (B. Lu and C. Gerard, unpublished data; however, a recent study by Li *et al.*[25] confirmed the presence of CXCR3 expression on mouse eosinophils in a parasite mode of Schistosome infection). Thus, we propose that CXCR3 expressed on eosinophils or T cells[26] may contribute to generating eosinophil infiltrates, or that CXCR3 expressed on endothelial cells[27] could be involved.

The source of Th1- and Th2-type cytokines in DTH

We found that both Th1 and Th2 cytokines are involved in early elicited eosinophil-rich DTH, but the exact source is uncertain. Thus, other cell types besides T cells could produce these cytokines in DTH. We hypothesize that Th0 memory cells producing both Th1- and Th2-type cytokines[28–30] may be responsible, for three reasons:

(1) The mixture of Th1 and Th2 cytokines mediating early DTH fits Th0 cells.
(2) Elicitation of DTH on day 3 postimmunization is probably too early for the full development of separate Th1 and Th2 cells,[29] especially considering the more natural *in vivo* conditions of immunization we used with KLH in saline that employed no adjuvants, added cytokines, or anti-cytokine skewing procedures that are used *in vitro* to direct T cells towards a Th1 or a Th2 phenotype.[29–31]
(3) Co-expression of Th1 and Th2 cytokines is consistent with a single cell source, as separate cells would be expected to be cross-inhibitory.

In fact, another study immunized mice s.c. with KLH in alum and found that the majority of day-7 *in vitro*-generated T-cell clones were Th0.[32] There are only a few previous observations suggesting an *in vivo* effector function of Th0 cells. One study showed that some Th0 clones produce a macrophage-acting procoagulant which is also produced by DTH-mediating Th1 cells, and thus postulated a DTH effector role of Th0 cells.[33] Furthermore, a recent study showed that a Th0 clone, derived from mice immunized with OVA protein without CFA, induced a mast cell-dependent form of DTH.[34] These findings are relevant to this study of early eosinophil-rich DTH induced without adjuvant, which is mast cell-dependent.[35] However, the actual demonstration that Th0 cells are responsible for early DTH remains to be accomplished in further studies.

Conclusions

We found that early DTH responses to soluble protein antigens, and also early CS responses, can be caused by a complex mixture of cells, cytokines and chemokines. Early elicited DTH is mediated by combined Th1- and Th2-type cytokines that we hypothesize may be derived from activated Th0 DTH-effector cells, perhaps as a transient early phase. It is well accepted that DTH mechanisms are relevant to clinical immune resistance to microbes and tumours, and also are important in organ-specific autoimmunity and some allergies. Apparent Th1/Th2 mixtures in more chronic lesions may be a mixture of Th1- and Th2-type cytokine responses that also could be mediated by Th0 cells.

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Immunogenicity and protective efficacy of a formalin-inactivated rotavirus vaccine combined with lipid adjuvants

Kari Johansen [a,b,*], Ulf Schröder [c], Lennart Svensson [a]

[a] *Department of Virology, Swedish Institute for Infectious Disease Control, Karolinska Institute, S-171 82 Solna, Sweden*
[b] *Department of Women and Child Health, Karolinska hospital, S-171 77 Stockholm, Sweden*
[c] *Department of Bacteriology, Swedish Institute for Infectious Disease Control, Karolinska Institute, S-171 82 Solna, Sweden*

Abstract

The immunogenicity and protective efficacy of inactivated rotavirus vaccine administered intramuscularly with lipid adjuvants; MPL® (monophosphoryl lipid A from *Salmonella minnesota*) or L3® (monooleate/lauric acid) was evaluated in an infant mouse model. Purified and formalin-inactivated rhesus rotavirus (I-RRV) combined with one of the adjuvants were administered to female balb/c mice at 0, 4 and 8 weeks. High serum IgG antibody titers developed in all vaccinated groups; I-RRV (GMT 45,524 ± 9,819), I-RRV-MPL® (GMT 190,637 ± 64,250) and I-RRV-L3® (GMT 126,266 ± 27,553). The formalin-inactivation procedure preserved neutralizing epitopes and elicited high neutralizing antibody titers; I-RRV (GMT 43,053 S.E.M. ± 4,189), I-RRV-MPL® (GMT 66,398 S.E.M. ± 20,202) and I-RRV-L3® (GMT 60,887 S.E.M. ± 10,750). All offsprings to immunized dams were protected against clinical diarrhea upon oral challenge with RRV. The IgG1/IgG2a ratio was in all immunized groups ~1 suggesting development of a balanced Th1/Th2 response.

Keywords: Rotavirus; Parenteral immunization; Lipid adjuvants; Mice

1. Introduction

Rotavirus is the most important cause of severe gastroenteritis in young children and is estimated to be responsible for ~600,000 deaths per year worldwide [1]. Rotavirus infections occur in children throughout the world regardless of socioeconomic background and an effective rotavirus vaccine early in life would have a beneficial effect on child morbidity and mortality. Several strategies have been employed to develop a rotavirus vaccine; including live simian-human reassortment strains (RRV-TV), live bovine-human reassortment strains (W179-9 and D × UK), live attenuated human serotype 1 strain (89-12), live neonatal human serotype X strain (RV3), subunit vaccines (VP4 or VP7), naked DNA and virus-like particles [2–9]. Five oral live attenuated rotavirus vaccines are presently in clinical trials for use in children. The three vaccine candidates; quadrivalent rhesus rotavirus (RRV-TV), bovine-human rotavirus (W179-9) and human serotype 1 (89-12) have been shown to provide a protection rate 63–89% against subsequent rotavirus infections [3,9,10]. Two other vaccine candidates (RV3 and d × UK) have so far only been tested in phase 1 studies with promising results [2,5].

Since the oral vaccine candidates provide good but not complete protection against rotavirus diarrhea; the possibility of parenteral immunization with inactivated rotavirus has been given serious consideration by several investigators. In five animal models, the infant mouse model, the adult mouse model, the rabbit model, the bovine model and the equine model partial or complete protective immunity has been induced with inactivated rotavirus. In the infant mouse model Sheridan et al. reported prevention of rotavirus-induced diarrhea in neonatal mice born to dams immunized with empty capsids of simian rotavirus SA-11 [11]. Furthermore, Offit and Dudzik showed protection in infant mice born to dams immunized with β-propriolactone- and UV-inactivated rhesus rotavirus (RRV) [12]. In the adult mouse model McNeal et al. reported on partial protection, evaluated by virus shedding, after intraperitoneal immunization with UV-inactivated rotaviruses and subsequent challenge with epizootic diarrhea of infant mice (EDIM) [13] and more recently Coffin et al. reported that psoralen-inactivated EDIM provided partial protection upon subsequent EDIM exposure [14]. In the rabbit model, Conner et al. showed that formalin-inactivated SA 11 rotavirus provided protective immunity after challenge with rabbit rotavirus, evalu-

* Corresponding author. Tel.: +46-8-457-2694; fax: +46-8-337272.
E-mail address: kari.johansen@smi.ki.se (K. Johansen).

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ated by virus shedding [15]. In an early study Castrucci et al. reported partial protection against diarrheal symptoms, but complete protection against mortality in calves fed immune colostrum from cows immunized with formalin-inactivated bovine strain 81/36F [16] and recently Barrandeguy et al. noted partial protection in foals by parenteral immunization with an inactivated combined vaccine including SA11, H2 and Lincoln with aluminum hydroxide as adjuvant [17]. However, in a sixth model using piglets Yuan et al. reported contrasting results [18]. Piglets immunized with binary ethyleneimine-inactivated human rotavirus (Wa) were not protected against clinical diarrhea upon challenge with virulent Wa virus. To further optimize the immune response with parenteral immunization, McNeal et al. recently presented two studies in the adult mouse model on the effect of several potent adjuvants with potential for human use; QS-21, QS-7, QUIL A, PCPP and RAS. QS-21 and PCPP together with UV/psoralen-inactivated EDIM were shown to induce the best immune response and provided partial protection evaluated by virus shedding [19,20].

In this study, we investigated the immunogenicity and protective efficacy against diarrhea by parenteral immunization with formalin-inactivated rotavirus combined with lipid adjuvants. Formalin-inactivated rotavirus (I-RRV) was administered alone or in combination with one of two lipid adjuvants; monophosphoryl lipid A from *Salmonella minnesota* (MPL®) [21] or monooleate/lauric acid (L3®) [22]. Formalin-inactivated RRV with either one of the two lipid adjuvants were in this study shown to be immunogenic and induce protection against diarrhea in the infant mouse model.

2. Materials and methods

2.1. Virus and cells

Confluent monolayers of foetal rhesus monkey kidney cells (Ma 104) were washed twice with Eagle's MEM without serum and infected with trypsin-activated rhesus rotavirus (RRV) at a m.o.i. of 5. After 1 h adsorption at 37 °C the virus inoculum was washed off and Eagle's MEM containing 0.5 μg/ml trypsin (Sigma) without serum was added. The cells were harvested 2–3 days post-infection and froze-thawed three times. After centrifugation at $12,000 \times g$ for 10 min the cell debris was discarded and the supernatant was layered on top of a sucrose-CsCl gradient. Centrifugation at $100,000 \times g$ for 3 h resulted in the formation of two bands at a boyant density of 1.36 and 1.38 g/ml, respectively. By electron microscopy the bands represented triple-layered and double-layered virus particles. The triple-layered virus particles were harvested and washed five times on Centricon-30 concentrators (Amicon, MA, USA) with 150 mM NaCl, 10 mM Tris–HCl (pH 7.5) and 1 mM $CaCl_2$ (dialysis buffer). After virustitration on Ma 104 cells the virus particles were inactivated with 0.01% formalin in PBS for 72 h at RT. After completed inactivation formalin was washed away with dialysis buffer five times on Centricon-30 concentrators. Remaining infectivity was excluded by virus titration on Ma 104 cells starting with trypsin-activation of undiluted material and a 1:10 when added to the Ma 104 cells. Before immunization the morphology of the virus was checked by electron microscopy (Philips CM100) to ensure that the virus preparation contained triple-layered particles. Only batches with >90% of triple-layered particles were accepted for the vaccine study. RRV infected cell lysates were also used for the ELISA assays and in the virus neutralization assay.

2.2. Metabolic labeling

Radiolabeling of intracellular rotavirus proteins was performed essentially as previously described [23]. Briefly, confluent monolayers of Ma 104 cells were either infected with trypsin-activated RRV or mock-infected. After 60 min at 37 °C the inoculate was removed and Eagle's MEM was added. Eight hours post-infection medium was replaced with methionine-free Eagle's MEM to starve the cells for 1 h followed by labeling with ^{35}S methionine (500 mCi) (Amersham Laboratories, Buckinghamshire, England) for 3 h at 37 °C. The supernatant was then removed and cells were washed three times and then lysed in RIPA buffer (10 mM Tris, 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 2% Triton X-100, pH 7.8). The lysate was then centrifuged for 10 min at $12,000 \times g$ and the supernatant collected and stored at −70 °C until use.

2.3. Radioimmunoprecipitation assay

Radioimmunoprecipitation was performed essentially as previously described [23]. Briefly, 2 μl of mice serum was mixed with 20 μl of radiolabeled rotavirus-infected or mock-infected lysate in a total volume of 500 μl of RIPA buffer. After 18 h of incubation at +4 °C 25 μl of Staphylococcus aureus protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in PBS was added for 1 h at room temperature. Beads were then washed five times with RIPA-buffer and finally with 0.01 M Tris–HCl, 0.15 M NaCl, pH 8.0. After the last wash 40 μl of sample buffer (3% SDS, 3% 2-mercaptoethanol, 0.1% EDTA, 10% v/v glycerol in 62 mM Tris-H3PO4, pH 6.8) were added and the samples were boiled for 5 min. Samples were then separated by SDS-PAGE (11%) and gels were fixed in 10% (v/v) glacial acetic acid and 35% (v/v) methanol. Gels were then processed for fluorography by soaking in Amplify (Amersham Laboratories, Buckinghamshire, Great Britain) for 30 min, dried and exposed to a Kodak X-Omat AR-5 film at −70 °C before development.

2.4. Mice

Rotavirus-antibody-negative 6 weeks old female SPF balb/c mice purchased from B & K Laboratories, Sollentuna,

Sweden were housed in individual cages. Two weeks after completion of the vaccination schedule, dams were made pregnant and virus challenge was performed in their offsprings. For the rotavirus challenge of infant mice, the newborn mice with their mother were moved on the day of challenge to a physically separated animal facility to avoid any possible contamination. Offsprings to immunized dams were on days 3–5 of life challenged orally with live RRV (3×10^7 pfu/mouse) and evaluated for clinical diarrhea by daily abdominal palpation for 6 days [24].

2.5. Parenteral immunization and adjuvants

Mice were immunized in the quadriceps femoris muscle (i.m.) with formalin-inactivated virus (I-RRV 2×10^8 pfu/dose before inactivation) alone, or in combination with one of three adjuvants monophosphoryl lipid A from *Salmonella minnesota* (MPL® RIBI, Immunochem Research Inc., MT, USA), a lipid suspension containing 0.02% monooleate and 0.08% lauric acid (L3®, Eurocine, Stockholm, Sweden) or Freunds complete/incomplete adjuvant (Sigma, St. Louis, MO, USA) in a total volume of 100 ul. Mice were inoculated at 0, 4 and 8 weeks. In addition one group of mice were immunized intramuscularly with live RRV.

2.6. Sample collection and processing

Serum and fecal samples were obtained 2 weeks after each immunization and stored at −20 and −70 °C, respectively, until analysis. Fecal samples were prepared as follows before analysis. Two fecal pellets collected from individual mice were mixed with cold 0.5 ml 0.1% Tween 20–0.5% BSA-PBS and centrifuged at 12,000 × *g* for 5 min to remove fecal solids before analysis of rotavirus specific antibodies. Processed fecal samples were stored at −70 °C.

2.7. Detection of rotavirus specific antibodies

Postvaccination sera and fecal samples were tested for rotavirus-specific antibodies. All ELISAs were performed in 96-well plates (Costar) coated with rabbit-anti-rotavirus diluted 1/1000 in carbonate-bicarbonate buffer (pH 9.6) over night at 4 °C. Plates were then blocked with 0.1% BSA-PBS for 1 h at 37 °C. Following each step after the block, the plates were washed five times with 0.05% Tween 20–0.9% NaCl. The substrate used in all assays was TMB (ICN, Biochemicals, Cleveland, OH, USA) except when otherwise stated. The substrate was allowed to react at room temperature, and the reaction was stopped by the addition of 50 μl of 2 M H_2SO_4. Optical densities (OD) at 450 nm were determined with a Flow Titertech ELISA reader (Flow Laboratories Inc., McLean, VA, USA). A sample was considered positive if the optical density was greater than or equal to two times a pooled negative serum or fecal control. Antibody titers were defined as the reciprocal of the highest dilution giving a net OD value greater than or equal to 0.1.

2.7.1. Determination of rotavirus specific IgG and IgA antibody in serum

Coated plates as above were incubated with a lysate of RRV-infected cells for 1 h at 37 °C, sera were then diluted in two-fold dilutions starting at 1:100 and plates were incubated for 2 h at 37 °C. An HRP-conjugated goat-anti-mouse IgG (Biorad, Richmond, CA, USA) and rabbit-anti-mouse IgA (Cappel, Organon Teknika Corp., West Chester, PA, USA) was used in a dilution of 1:20,000 and 1:10,000, respectively, for 2 h at 37 °C.

2.7.2. Determination of rotavirus specific IgA and IgG antibody in fecal samples

Measurement of IgA and IgG in fecal samples was performed essentially as for the serum antibody ELISA with the following modifications; fecal samples were diluted in two-fold dilution steps starting at 1:2, and added to the plates and incubated over night at +4 °C.

2.7.3. Determination of rotavirus specific IgG1/IgG2a ratio

The immunoglobulin G isotypes IgG1 and IgG2a were determined to obtain an indication of a cell-mediated immune response being elicited. The ELISA was performed in a manner identical to that for the rotavirus specific serum IgG with the exception of alkaline-phosphatase-labeled rabbit anti-mouse IgG1 and IgG2a diluted 1:1000 as conjugate (Harlan Seralab Ltd., England) and OPD as substrate. The optical density was determined at 492 nm.

2.8. Detection of rotavirus specific neutralizing antibodies

Neutralizing antibodies to RRV were analyzed by a virus peroxidase focus reduction assay as previously described [25]. Briefly, serum or fecal samples diluted in two-fold dilutions were mixed with an equal volume of 1×10^3 pfu of trypsin-activated RRV and incubated for 1 h at room temperature. The mixture was added to Ma 104 monolayers for 1 h at 37 °C, cells were then washed and incubated at 37 °C. After 18 h of incubation cells were fixed in 2% paraformaldehyde in PBS and then after permeabilization with 1% Triton X-100 in PBS for 15 min infected cells were stained with a monoclonal antibody diluted 1:100 directed against rotavirus VP6 (255) for 1 h at 37 °C. A peroxidase-labeled goat-anti-mouse (Bio-Rad, Richmond, CA, USA) diluted 1:1000 was used as conjugate and 3-amino-ethylcarbazole (Sigma, St. Louis, MO, USA) in 0.05 M sodiumacetate buffer containing 0.01% H_2O_2 was used as substrate. The neutralizing titers were defined as the reciprocal of the serum dilution showing 60% reduction in the number of infected cells.

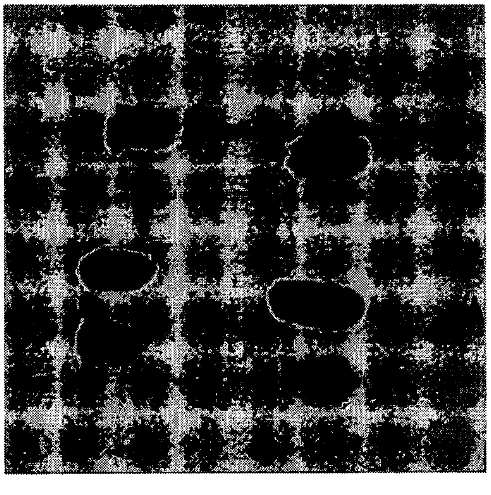

Fig. 1. Radioimmunoprecipitation of sera from two balb/c mice immunized intramuscularly with formalin-inactivated RRV. 2 μl of mice serum was mixed with 20 μl of radiolabeled rotavirus- or mock-infected cellysate in a total volume of 500 μl of RIPA-buffer. After 24 h of incubation at +4 °C, 25 μl of *Staphylococus aureus* protein A-Sepharose CL-4B in PBS was added for 1 h at room temperature. Beads were then washed and after last wash 40 μl of sample buffer was added and samples were boiled for 5 min. Samples were then separated by SDS-PAGE (11%) and gels were fixed and processed for fluorography. Lane 1: Molecular weight markers (200, 92, 69, 46 and 30 K). Lanes 2 and 4: Serum from mice nos. 1 and 2 immunoprecipitated with ^{35}S-rotavirus infected cellysate. Lanes 3 and 5: Serum from mouse nos. 1 and 2 immunoprecapitated with ^{35}S-mockinfected cell lysate. Antibodies to VP1, VP2, VP4, VP6 and VP7 evolved but not to non-structural proteins.

3. Results

3.1. Characterization of rotavirus antigen

The infectivity of the virus preparation was reduced from 2×10^9 pfu/ml to below the detection limit ($<10^1$ pfu/ml) by formalin treatment. Electron microscopy after purification and inactivation ensured complete virus particles. During establishment of purification and inactivation procedures immune responses in mice were examined by radioimmunoprecipitation to exclude development of antibodies to non-structural proteins, thus verifying by another method that the established procedure of inactivation was effective (Fig. 1). It was also noted by radioimmunoprecipitation assay that an immune response developed predominantly to VP 2, VP4, VP6 and VP7, which are the four structural proteins that induce the strongest immune response also in naturally infected children and VP4 and VP7 are known to induce neutralizing antibodies (Fig. 1) [26,27].

3.2. Parenteral immunization with formalin-inactivated RRV in combination with lipid adjuvants induce high serum IgG antibody titers

Parenteral administration of formalin-inactivated rotavirus (2×10^8 pfu/dose) combined with lipid adjuvants induced high IgG titers (Table 1) with the order of IgG antibody titers I-RRV-MPL® > I-RRV-L3® > I-RRV only. While the two lipid adjuvants in combination with I-RRV induced higher IgG antibody titers, the titers were lower than with Freunds adjuvant, but similar to live RRV. A significant IgG antibody titer rise (>four-fold rise) was clearly seen between the first and second dose for all vaccine variants tested, but only a modest to no rise was seen between the second and third dose for most variants (Table 1). Low or undetectable serum IgA titers were detected after each immunization. Fecal IgA and IgG titers were modest in all vaccinated animals, with titers in the animals that received inactivated virus alone or lipid adjuvants slightly lower than in mice that received inactivated rotavirus with Freunds adjuvant or live rotavirus (Table 1).

3.3. IgG1/IgG2a ratio after one and three immunizations

The subclasses IgG1 and IgG2a were analyzed to evaluate the ability of the different vaccine variants to induce humoral

Table 1
Rotavirus antibody titers following three parenteral immunizations of mice with formalin-inactivated RRV (I-RRV) in combination with two lipid adjuvants; MPL® or L3®[a]

Vaccine	Adjuvant	Route of administration	Rotavirus specific serum IgG antibodies after			Feacal IgG after	Feacal IgA after
			First immunization	Second immunization	Third immunization	Third immunization	Third immunization
I-RRV	–	i.m.	11,086 ± 2987[b]	45,524 ± 9818	45,524 ± 9819	8 ± 3	10 ± 2
I-RRV	MPL®	i.m.	3461 ± 1131	166,176 ± 23,862	190,637 ± 64,250	11 ± 4	9 ± 2
I-RRV	L3®	i.m.	10,660 ± 2651	82,364 ± 23,068	126,266 ± 27,553	5 ± 2	11 ± 5
I-RRV	Freunds	i.m.	50,546 ± 30,737	787,934 ± 307,565	787,934 ± 307,565	25 ± 19	10 ± 2
Live RRV	–	i.m.	24,300 ± 0	151,638 ± 30,737	151,638 ± 30 737	81 ± 14	8 ± 2
PBS	–	i.m.	<100	<100	<100	<2	<2

[a] Female balb/c mice (six animals/group) were immunized intramuscularly at 0, 4 and 8 weeks with formalin-inactivated RRV (2×10^8 pfu/mouse), either with or without adjuvant. Serum samples were collected 2 weeks after each immunization and were analyzed for rotavirus specific antibodies, starting at a dilution 1:100. Fecal samples were collected 2 weeks after the completed vaccination schedule of three doses and were analyzed for rotavirus specific antibodies starting at a dilution 1:2.

[b] The data are expressed as the reciprocal of serum and fecal antibody titer (GMT ± S.E.M.) concept.

versus cell-mediated immune responses. The ratio between IgG1 and IgG2a was found to be slightly less than 1 after first and third immunization for I-RRV, I-RRV-MPL® and for I-RRV-L3® (Fig. 2) suggesting an activation of a humoral and a cell-mediated immune response.

3.4. Formalin-inactivated RRV with lipid adjuvants induce high serum neutralizing antibody titers

All vaccine variants induced significant neutralizing antibody titers after three doses (Table 2). Both MPL® and L3® induced slightly higher neutralizing antibody titers than I-RRV alone and in the same range as Freunds adjuvant. Also the group receiving live RRV parenterally developed high serum neutralizing antibody titer. The kinetics of neutralizing antibodies were shown to differ between immunized groups. I-RRV-Freunds and live RRV induced already after the first immunization significantly higher neutralizing antibody titers as compared to I-RRV with or without the lipid adjuvants. However, already after two doses the differences were largely eliminated (Table 2). Titer increases were significant (>four-fold) for the I-RRV with lipid adjuvants after the second dose, but very little change was noted after the third dose. No neutralizing antibodies were identified in stool samples collected from immunized mice.

3.5. Parenteral immunization with formalin-inactivated RRV with MPL® or L3® induce complete protection against clinical diarrhea in offsprings

While previous studies, using the infant and the adult mouse models, have successfully shown that partial or complete protection against clinical diarrhea (infant mice) or virus shedding (adult mice), respectively, can be achieved by parenteral immunization with psoralen/UV inactivated rotavirus or virus-like particles, no information is available whether formalin-inactivated rotavirus can induce protection against clinical diarrhea in mice. To address this question

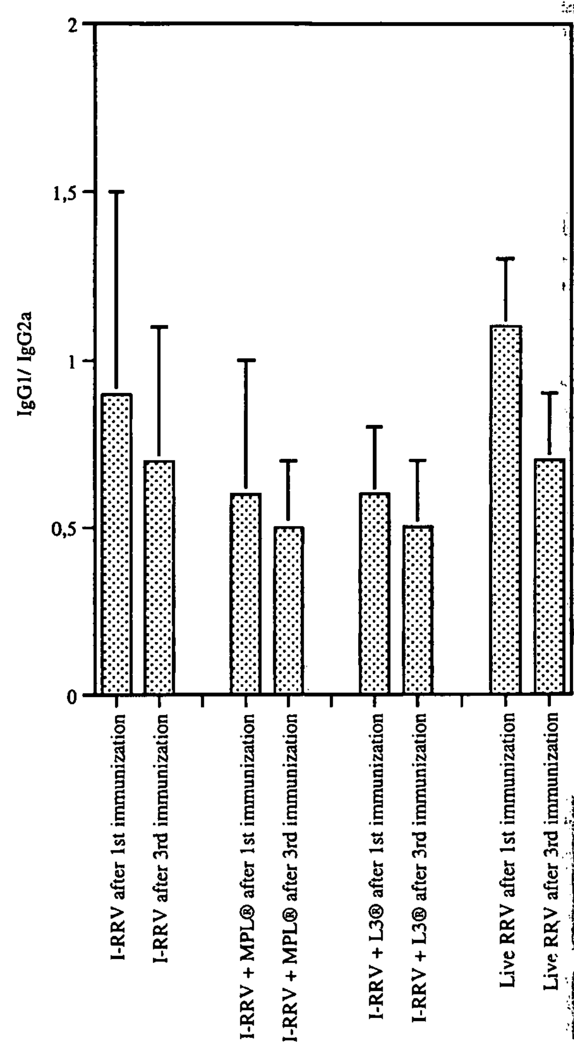

Fig. 2. IgG1/IgG2a ratio after first and third intramuscular immunization with formalin-inactivated RRV alone or in combination with MPL® or L3®.

Table 2
Protection and rotavirus specific serum neutralizing antibody titers following parenteral immunization of mice with formalin-inactivated RRV (I-RRV) in combination with MPL® or L3®[a]

Vaccine	Adjuvant	Route of administration	Serum neutralizing antibodies after			Protected offsprings[c]
			First immunization	Second immunization	Third immunization	
I-RRV	–	i.m.	5868 ± 1416[b]	25,600 ± 5272	43,053 ± 4189	49/49
I-RRV	MPL®	i.m.	4149 ± 705	39,480 ± 12,448	66,398 ± 20,202	52/52
I-RRV	L3®	i.m.	4935 ± 671	46,950 ± 3200	60,887 ± 10,750	40/40
I-RRV	Freunds	i.m.	25,600 ± 8112	81,274 ± 12,800	64,507 ± 15,647	34/34
Live RRV	–	i.m.	20,318 ± 8207	57,470 ± 12,800	57,470 ± 12,800	30/30
PBS	–	i.m.	<100	<100	<100	1/18

[a] Female balb/c mice (six animals/group) were immunized intramuscularly at 0, 4 and 8 weeks with formalin inactivated RRV (2×10^8 pfu/mouse) either with or without adjuvant. Serum samples were collected 2 weeks after each immunization and were analyzed for rotavirus neutralizing antibodies starting at a dilution 1:100.

[b] The data are expressed as the reciprocal of the serum dilution showing a 60% reduction in number of RRV infected cells (GMT ± S.E.M.).

[c] Offsprings to immunized dams were on days 3–5 of life challenged orally with live RRV 3×10^7 pfu/mouse and evaluated for clinical diarrhea by daily abdominal palpation for 6 days.

immunized dams were made pregnant and their newborn offsprings were challenged orally at days 3–5 with 3×10^7 pfu of RRV and evaluated for diarrhea. In contrast to offsprings to naive dams, offsprings to immunized dams were completely protected against clinical diarrhea (Table 2). No difference in protection was found between offsprings to dams immunized with and without adjuvants, inspite of the slightly higher neutralizing antibody response seen in dams immunized with adjuvants. In the control group 17/18 mice developed diarrhea for a mean time of 2.7 days.

4. Discussion

The protective efficacy of a natural rotavirus infection against subsequent symptomatic rotavirus infections is estimated to be 58–75% [28–30] and a similar protective efficacy has been observed with the current live oral rotavirus vaccine candidates; 63–89% [3,9,10]. Since the evaluated oral vaccine candidates provide good but only partial protection against subsequent infection, development of a parenteral inactivated vaccine has been given serious consideration by several investigators. Recently, a severe adverse reaction in the form of intussusception has been reported among recipients of the oral tetravalent rhesus rotavirus vaccine, adding to an increased interest for a parenterally administered rotavirus vaccine [31]. For decades parenteral administration has been used to stimulate protection against microbes, whose site of entry, and for some pathogens replication, is limited to the mucosal surfaces. This includes vaccines against influensa virus, poliovirus, hepatitis A virus and *Bordetella pertussis*. In addition to provide protection from mucosal disease, parenteral administration has also been shown to limit virus shedding in feces among inactivated poliovirus vaccine recipients following exposure to wildtype poliovirus [32]. Early and more recent studies with inactivated rotavirus have also shown in various animal models that parenteral immunizations can provide partial or complete protective immunity [11,12,14,15,19,20].

The objective of this study was to examine whether parenteral administration of formalin-inactivated rotavirus combined with lipid adjuvants; MPL® or L3® [21,22] could induce protection from clinical diarrhea. In this study preservation of the antigenic structure of the virus particles was given great consideration. Complete protection from clinical diarrhea was obtained with formalin-inactivated rotavirus alone and in combination with MPL® or L3®. Since serum IgA titers were low or undetectable, and fecal IgA titers only modest, it seems reasonable to assume that serum IgG and possibly fecal IgG was a mediator of protection. In a recent study by O'Neal et al. it was shown in IgA knockout mice that fecal or systemic IgA is not essential for protection against rotavirus infection and suggest that in the absence of IgA, IgG may play a significant role in protection from mucosal patogens [33]. Also Coffin et al. provide evidence for that parenteral administration of live rotavirus may induce mucosal immunity by migration of antigen-presenting B cells from peripheral to mucosal lymphoid tissues [34].

Administration of inactivated RRV without adjuvants resulted in similar protection rates as vaccination with adjuvants. A reasonable explanation to this may be that the vaccination dose was high enough to mount a protective immune response in the absence of any adjuvant.

Several adjuvants have earlier been shown to provide a stimulatory effect with inactivated rotavirus vaccines [15,20]. The two adjuvants tested here; MPL® and L3®, were chosen since they are easily mixed with the vaccine preparation without any loss of antigen or its immunogenicity. Suspensions of monoglycerides and fatty acids have been shown to stimulate an immune response comparable to Alum adsorbed antigens [22] but has not previously been tested with rotavirus. A variant of MPL®, RAS, has earlier been tested with UV/psoralen inactivated EDIM given parenterally to adult mice by McNeal et al. with development of high serum IgG antibody response, low neutralizing antibody response, modest fecal IgG and IgA response and partial protection noted as virus shedding [20]. In our study MPL® was found to induce high serum IgG antibody titers, high serum neutralizing antibody titers, modest fecal and IgA and IgG antibody titers and complete protection against clinical diarrhea in offsprings. Similar results were obtained with monooleate/lauric acid suspension. The higher neutralizing antibody titers that evolved in our study with all vaccine variants in comparison to previous studies, could possibly be attributed to the high dose of antigen (2×10^8 pfu/dose) and its preserved antigenic structure, provided by the inactivation method. Commonly used methods of inactivation are β-propriolactone inactivation, psoralen inactivation, UV-inactivation and binary ethyleneimine-inactivation [12,13,18]. Their capacity to induce inactivation is well documented, but less is known on how well the outer proteins are preserved. Offit and Dudzik noted that treatment with 0.15% β-propriolactone altered the structural integrity of outer-capsid proteins as indirectly reflected in decreased viral hemagglutinating activity, but they concluded since neutralizing antibodies evolved and protective immunity was obtained, that sufficient amounts of the outer capsid proteins must have been preserved [12]. McNeal et al. showed that UV/psoralen inactivation of purified EDIM particles analyzed by SDS-PAGE and stained with Coomassie brilliant blue still after inactivation had protein bands corresponding to VP4 and VP7, although weaker than before [19]. Other investigators using formalin-inactivation have also studied the preservation of the antigenic structure. Conner et al. found that the reactivity of formalin-inactivated SA 11, with in total 10 monoclonal antibodies to VP4 and VP7, differed significantly only with one monoclonal antibody to VP7 [15] while Zissis et al. reported that formalin-inactivation of RIT 4237 caused alterations of the virus structure, which later failed to induce cross-protection of piglets challenged with human rotavirus [35]. However, formalin-inactivation is today, in contrast

to most other inactivation methods, accepted for human use and used for preparation of parenteral hepatitis A and poliovirus vaccines.

The kinetics of development of serum IgG and neutralizing antibodies suggest that two immunizations provide good rotavirus specific serum IgG antibodies and neutralizing antibodies. We vaccinated dams with both live and inactivated rotavirus to determine whether the level of protection or antibody response differed. The most obvious difference between these groups was the significant fecal IgG response noted after vaccination with live virus but also the neutralizing antibody response developed earlier with live rotavirus.

The subsets of antibodies stimulated by immunization is dependent on the expression of T helper subsets. T helper lymphocytes (CD4+) can be divided into two distinct effector cell subsets, based on functional capabilities and cytokine profiles [36]. Th1 CD4+ T cell clones have been shown to secrete IL-2, IFN, TNF and induce IgG2a isotype production [37]. Th2 clones have been shown to secrete IL-4, IL-5, and induce IgG1 isotype production [38]. The IgG1/IgG2a ratio has gained recognition for evaluating whether a Th1 or Th2 immune response develop after immunizations [20,39]. In this study the highest ratio was noted for live RRV, while inactivated RRV alone or combined with adjuvants were shown to be ~1. This indicates that the various vaccine variants tested induced a balanced Th1 and Th2 response. The IgG1/IgG2a ratio decreased between the first and third immunization slightly favoring a Th1 response. These results are similar to what other investigators using inactivated rotavirus particles alone, rotavirus particles with adjuvants such as QS 21, PCPP, QUIL A or virus-like particles administered parenterally have seen [14,21]. These results differ from when aluminum hydroxide is used as adjuvant, where a Th2 response is strongly favored [39].

A parenteral vaccine might overcome some of the limitations observed with an oral live rotavirus vaccine. Preexisting maternal antibody in children given oral live rotavirus vaccine, can interfere with vaccine take. Development of reassortments between vaccine strains and wild-type strains is avoided. Last but not least, if the newly reported adverse effect of intussusception in oral vaccine recipients is statistically confirmed to be correlated to oral administration, the use of an inactivated virus parenterally would be further explored.

The mechanism and proteins involved in mediating protective immunity against rotavirus disease has long been a matter of debate. The non-structural NSP4 protein and VP6 have both recently been suggested to be associated with protective immunity in mice [40,41]. However, it is generally accepted that immune responses to the outer capsid proteins VP4 and VP7 play an important role in preventing the host from clinical diarrhea [42]. The fact that the inactivated and non-replicating virus did not stimulate any detectable immune responses to non-structural proteins suggests that immune responses against non-structural proteins are not required for preventing clinical diarrhea in mice.

We conclude that formalin-inactivated rotavirus given intramuscularly with MPL® or L3® stimulated a potent immune response with high neutralizing antibody titers and induced protective immunity in this infant mouse model. Future studies should consider the possibility of lowering the dose of virus and reducing the number of vaccine doses.

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Mechanisms of Monophosphoryl Lipid A Augmentation of Host Responses to Recombinant HagB from *Porphyromonas gingivalis*

Qiu-Bo Yang,[1] Michael Martin,[1] Suzanne M. Michalek,[1,2] and Jannet Katz[2]*

Department of Microbiology[1] and Department of Oral Biology,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294

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***Porphyromonas gingivalis*, a gram-negative, black-pigmented anaerobe, is among the microorganisms implicated in the etiology of adult periodontal disease. This bacterium possesses a number of factors, including hemagglutinins, of potential importance in virulence. Our laboratory has shown the induction of protection to *P. gingivalis* infection after subcutaneous immunization with recombinant hemagglutinin B (rHagB). The purpose of this study was to determine if humoral antibody responses are induced after intranasal (i.n.) immunization of rHagB and if monophosphoryl lipid A (MPL), a nontoxic derivative of the lipid A region of lipopolysaccharide, acts as a mucosal adjuvant and potentiates responses to rHagB. Further, the effects of MPL on the nature of the response to HagB and on the costimulatory molecules B7-1 and B7-2 on different antigen-presenting cells (APC) were evaluated. Groups of BALB/c mice were immunized three times (2-week intervals) by the i.n. route with HagB (20 μg) alone or with MPL (25 μg). A group of nonimmunized mice served as control. Serum and saliva samples were collected prior to immunization and at approximately 2-week intervals and evaluated for serum immunoglobulin G (IgG) and IgG subclass and for salivary IgA antibody activity by enzyme-linked immunosorbent assay. Mice immunized with rHagB plus MPL had significantly higher salivary IgA ($P < 0.05$) and serum IgG ($P < 0.05$) anti-HagB responses than mice immunized with rHagB alone. The IgG1 and IgG2a subclass responses seen in mice immunized with rHagB plus MPL were significantly higher ($P < 0.05$) than those seen in mice immunized with rHagB only. Further, the IgG2a/IgG1 ratio in the latter group was ≈1, whereas in mice immunized with rHagB plus MPL the ratio was <1. These results provide evidence for the participation of T helper (Th) 1 and Th2 cells in responses to rHagB and that MPL potentiates a type 2 response to HagB. MPL was also shown to preferentially up-regulate B7-2 expression on B cells, whereas a preferential increase in B7-1 costimulatory molecule was seen on macrophages and dendritic cells. These results provide evidence that MPL exerts a differential regulation in the expression of costimulatory molecules on APC.**

Periodontal disease is the result of interactions between periodontal pathogens such as *Porphyromonas gingivalis* and the host's immune system. Interest in developing a vaccine against periodontitis has recently increased not only because about 25% of the adult population is affected by this infectious disease but also because of the possibility of an association between periodontitis and systemic diseases (3, 9, 48). Immunization studies with *P. gingivalis* whole cells or purified antigens in animal models have provided encouraging results that indicate a vaccine can be developed to protect against periodontal disease (27, 47, 54, 60).

Several virulence antigens of *P. gingivalis* have been identified, such as fimbriae, hemagglutinins, lipopolysaccharide (LPS), and proteases (23). The fimbriae and hemagglutinins appear to be involved in the attachment of *P. gingivalis* to host tissues (11, 22, 32, 51, 61). A number of hemagglutinins have been identified and their genes have been cloned (16, 36–38, 50, 51). Although evidence for a direct role of the hemagglutinins in host tissue binding has not yet been demonstrated, we have previously shown in an experimental rat model that systemic immunization with recombinant hemagglutinin B (rHagB) results in protection from *P. gingivalis* infection (27). These results suggest a role for HagB in periodontal disease pathogenesis.

Vaccines consisting of antigen alone are often not very effective in inducing the desired immune responses. Therefore, adjuvants are commonly used to enhance the host response to the vaccine antigen. Adjuvants can alter the avidity, affinity, kinetics, and specificity of the antibody response to the antigen, as well as affecting cell-mediated immunity (12, 25). Thus, it is essential to elucidate the cellular mechanisms by which adjuvants modulate host responses to an antigen. Monophosphoryl lipid A (MPL) is a detoxified derivative of the LPS of *Salmonella enterica* serovar Minnesota R595 that lacks the endotoxic properties but retains both the adjuvant and immunostimulatory activities of the parent LPS (5, 19, 46). Studies in humans have shown that systemic coadministration of MPL and antigen results in an increased immune response to the specific antigen without causing toxicity (56, 58). Although most studies with MPL have involved the systemic route of immunization, it has recently been shown to also be a mucosal adjuvant (2, 8, 42, 53). However, the mechanism(s) involved in MPL adjuvanticity has not been fully defined. MPL has been shown to induce interleukin-12 (IL-12) protein and IL-10 mRNA production (43, 52). It has also been suggested to exert an effect on the costimulatory molecules B7-1 (CD80) and B7-2

* Corresponding author. Mailing address: Departments of Microbiology and Oral Biology, University of Alabama at Birmingham, 845 19th St. South, BBRB 713/5, Birmingham, AL 35294-2170. Phone: (205) 934-2878. Fax: (205) 934-1426. E-mail: jenny_katz@micro.microbio.uab.edu.

(CD86), i.e., to induce B7-1 but not B7-2 expression on monocytes (10).

T-cell activation requires the recognition of the T-cell receptor (TCR) with the major histocompatibility complex (MHC)-peptide complex on antigen-presenting cells (APC) and the interaction between costimulatory molecules on APC and their respective receptors on T cells (35, 55). The receptor CD28 on T cells interacts with the costimulatory molecules B7-1 and B7-2 on APC (1, 40, 41). In the absence of costimulation, antigen-specific hyporesponsiveness, clonal T-cell anergy, or apoptosis may occur (6, 41). The CD28 receptor and the B7 ligands are type I transmembrane glycoproteins and are members of the immunoglobulin superfamily (20). Both B7-1 and B7-2 are induced by cell activation; however, they respond to different stimuli and exhibit different expression kinetics and receptor binding properties (26, 39). These molecules are mainly expressed on monocytes, dendritic cells, and activated B cells. The B7-1 and B7-2 costimulatory molecules have been shown to influence the immune response by skewing the $CD4^+$ cell response towards either a Th1 or Th2 cell differentiation. Studies in a murine experimental autoimmune encephalitis model have shown that antibodies to B7-1 increased IL-4 (Th2 derived), while antibodies to B7-2 enhanced gamma interferon (IFN-γ) (Th1 derived) production (31). Moreover, in vitro studies with human T cells have shown that B7-2 transfectants preferentially activate type 2 cytokines, whereas B7-1 transfectants produced type 1 cytokines (14). However, other investigations have found that both B7-1 and B7-2 were involved in IL-4 and IFN-γ production in the mouse system (33).

The purpose of the present study was to determine the effectiveness of MPL as a mucosal adjuvant in potentiating a specific antibody response to the *P. gingivalis* HagB antigen following intranasal (i.n.) immunization of mice with rHagB. Furthermore, the involvement of the costimulatory molecules B7-1 and B7-2 on the various APC populations in the adjuvant activity of MPL was investigated.

MATERIALS AND METHODS

HagB purification. HagB was purified as previously described (29) with some modifications. Briefly, the *hagB* gene was cloned from *P. gingivalis* 381 into a pET vector with a *lac* promoter and histidine tag and expressed in *Escherichia coli* JM 109 (kindly provided by Ann Progulske-Fox and Thomas Brown, University of Florida, Gainesville). A culture of *E. coli* (≈30 ml) was grown overnight at 35°C in Luria-Bertani (LB) broth containing carbenicillin and kanamycin. The overnight culture was transferred to LB broth (2 liters) with antibiotics and incubated at 35°C with vigorous shaking. When the concentration of bacteria reached an optical density at 600 nm of 0.6, 0.36 mM isopropyl-β-D-thiogalactoside was added and the culture was incubated for an additional 3 h. The culture was centrifuged and the pellet was resuspended in 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and stored at −70°C. The next day, the cells were thawed at room temperature and sonicated (10 s) three times at 5-s intervals. The suspension was centrifuged at 34,900 × *g* for 15 min, and the supernatant was passed through a 0.45-μm-pore-size low protein binding filter. rHagB was purified from the supernatant by using a modification of a previously described method (27). Briefly, the supernatant was allowed to flow through a His-bind resin column (Novagen, Madison, Wis.) which was precharged with 50 mM $NiSO_4$ and equilibrated with 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. The unbound protein was removed by washing the column with 40 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. The rHagB was recovered from the column by elution with 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. The fraction containing rHagB was dialyzed against phosphate-buffered saline (PBS). The purity of rHagB was confirmed by Western blot analysis using a rat anti-rHagB antibody (27). The concentration of rHagB was estimated by the bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.), using bovine serum albumin (BSA) as the standard.

MPL preparation. MPL-AF, an aqueous formulation containing MPL at a 4:1 molar ratio with dipalmitoylphosphatidyl choline and water, was obtained from Corixa Corporation (Hamilton, Mont.) and is referred to as MPL in this study.

Experimental design. Normal BALB/c mice used in this study were obtained from our breeding colony housed in the University of Alabama at Birmingham animal facilities. All mice were housed in covered cages and maintained under environment-controlled barrier conditions. All experiments were done according to National Institutes of Health guidelines, and protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Groups of normal female BALB/c mice (6 to 8 weeks old; six mice/group) were immunized three times on days 0, 14, and 28 by the i.n. route with either rHagB alone (20 μg; group A) or with a combination of MPL and rHagB (20 μg HagB plus 25 μg MPL; group B). A group of nonimmunized mice (group C) served as control. The vaccine was applied slowly into each nare. Serum and saliva samples were collected prior to immunization and at approximately 2-week intervals following the initial immunization. Briefly, saliva samples (≈100 μl) were collected over a 20-min interval after stimulation of the saliva flow by intraperitoneal injection of carbachol (5 μg in 0.05 ml). Saliva samples were clarified by centrifugation at 16,000 × *g* for 10 min at 4°C. The blood samples were collected from the retroorbital plexus by using heparinized capillary pipettes. The serum was obtained after centrifugation. All experimental samples were stored at −70°C until assayed. The levels of serum IgG, IgG subclasses, and salivary IgA anti-HagB antibody activity were determined by enzyme-linked immunosorbent assay (ELISA).

Evaluation of antibody responses. Antibody activity to rHagB in serum and saliva samples was assessed by ELISA as previously described (24, 27). Briefly, individual flat-bottom Maxisorp microtiter plates (Nunc International, Roskilde, Denmark) were coated with rHagB (1 μg/ml) or with optimal amounts of goat anti-mouse α or γ heavy chain antibody or affinity-purified goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Inc., Birmingham, Ala.) in borate buffer saline (BBS; 100 mM NaCl, 50 mM boric acid, 1.2 mM $Na_2B_4O_7$, pH 8.2). Nonspecific binding sites were blocked with 1% BSA and 0.01% sodium azide in BBS for 1 h at room temperature. From a starting dilution of serum (1:100 [groups A and C] and 1:500 [group B] for IgG, and 1:500 for IgG1 and 1:50 for IgG2a subclasses) or saliva (1:10 for specific or 1:100 for total IgA) prepared in BBS containing 1% BSA and 0.01% sodium azide, five twofold dilutions were added in duplicate to individual wells. After incubation (overnight at 4°C) and washing of plates, horseradish peroxidase-conjugated goat anti-mouse IgA, IgG, or IgG subclass antibody (Southern Biotechnology Associates, Inc.) was added to appropriate wells. After 4 h of incubation at room temperature, plates were washed and *o*-phenylenediamine substrate with hydrogen peroxide was added to wells. Color development was recorded at 490 nm. The concentrations of anti-rHagB antibodies in all serum and saliva samples were determined by interpolation on standard curves generated using a mouse immunoglobulin reference serum (ICN Biomedicals, Costa Mesa, Calif.) and constructed by a computer program based on a four-parameter logistic algorithm (Softmax/Molecular Devices Corp., Menlo Park, Calif.). Data were logarithmically transformed and statistical analysis was done by using the InStat program (GraphPad Software, San Diego, Calif.). The data were transformed and presented as the geometric mean ×/÷ the standard error of the mean (SEM) for ease of interpretation.

Proliferative responses. Single-cell suspensions were prepared from spleens obtained from normal BALB/c mice. Erythrocytes were removed by suspending the cells in lysis buffer (buffer ammonium chloride) for 5 min at 4°C. The spleen cells were then washed three times with Hank's balanced salt solution (Cellgro, Mediatech, Washington, D.C.). In addition, purified B cells were isolated from spleen cell suspensions by using CD43 MicroBeads (Miltenyi Biotec, Sunnyvale, Calif.). CD43 MicroBeads were incubated with the cell suspension for 15 min at 6°C in PBS containing 0.05% BSA and 2 mM EDTA. The cell suspension was then washed by adding a 20-fold excess of PBS containing 0.5% BSA and 2 mM EDTA. After centrifugation, cells were resuspended in 1 ml of PBS–0.5% BSA and added to the magnetic depletion column. $CD43^-$ B cells were eluted with 15 ml of PBS containing 0.5% BSA and 2 mM EDTA. Cells were then washed twice in PBS. The viability of the spleen cells and the purified B cells was assessed by trypan blue exclusion. The cells were then suspended in complete medium (RPMI 1640 [Cellgro] supplemented with 10% fetal bovine serum [FBS], 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g of sodium bicarbonate/liter, 20 mM HEPES, 50 μg of gentamicin/ml, 50 U of penicillin/ml, and 50 μg of streptomycin/ml) and added to sterile 96-well flat-bottom plates (Falcon Labware, Oxnard, Calif.) in triplicate at a concentration of 4 × 10^5 spleen cells or 2 × 10^5 B cells/well. Cells were incubated (37°C, 5% CO_2) with or

without various concentrations of MPL (0.001, 0.01, 0.1, 1, 2.5, 5, and 10 μg/ml) for 48 h (spleen cells) or 72 h (B cells). Cultures were pulsed with [^{3}H]TdR (Amersham Corp., Arlington Heights, Ill.) (0.5 μCi/well) during the last 18 to 20 h of incubation. Cells were harvested onto a glass fiber filter with a MASH II cell harvester (Microbiological Associates, Walkersville, Md.), and the amount of [^{3}H]TdR incorporation was measured in a liquid scintillation counter.

Flow cytometry. Single spleen cells, prepared as described above, were cultured at a concentration of 10^6 cells/ml in sterile 24-well plates (Falcon Labware) for 48 h in the presence or absence of MPL (0, 0.01, 0.1, 1, and 10 μg/ml). Cells were harvested by low-speed centrifugation and suspended in fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% FBS and 0.1% NaN_3). The B cells (B220$^+$) and macrophages (CD11b$^+$) were assessed for the presence of B7-1 (CD80) and B7-2 (CD86). Briefly, aliquots of the cell suspension (10^6 cells) were incubated with allophycocyanin-conjugated anti-B220 or -CD11b antibody for 30 min on ice. The cells were washed twice with FACS buffer and then aliquots of each preparation were incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD86 or phycoerythrin (PE)-labeled anti-CD80 antibody (Caltag Laboratories, Burlingame, Calif.) for 30 min on ice. The cells were washed, suspended in 2% paraformaldehyde, and analyzed using a FACStar flow cytometer (Becton Dickinson, Mountain View, Calif.). The B220$^+$ cells were further divided into B220^{+low} and B220^{+high}, since differences have been observed among these cell populations (45).

For the preparation of enriched dendritic cell cultures, spleens were teased apart using a 21-gauge needle and incubated with 1 mg of collagenase/ml and 20 μg of DNase/ml in complete RPMI 1640 medium for 30 min at 37°C. The single-cell suspension was then passed through a wire mesh (200 μm) and the leukocyte population was isolated by Histopaque (1083). The cells were washed three times with Hank's balanced salt solution, suspended in complete medium, and then plated in polypropylene tubes at ≈2 × 10^6/ml with or without MPL (2.5 μg/ml) for 24 h. The dendritic cells (CD11c-allophycocyanin conjugated; BD Pharmingen) were assessed for the presence of B7-1 (CD80) and B7-2 (CD86) as described above.

In vitro functional assay. Single spleen cell suspensions, prepared as described above, were separated into CD4$^+$ T cells and a T-cell-depleted population of APC. The CD4$^+$ cells were purified by using magnetized polystyrene Dynabeads coated with rat anti-mouse CD4 (L3T4) antibody (Dynal ASA, Oslo, Norway). Cells were incubated with the Dynabeads for 20 min at 4°C. Positive selection of CD4 cells was done with the Dynal magnetic particle concentrator. The resulting CD4$^+$ cell population was then washed three times in PBS containing 1% FBS and 2 mM EDTA. The purified CD4$^+$ cells were detached from the beads with Detachabead mouse CD4 antigen provided by the manufacturer, washed three times, and suspended in complete medium. The remaining spleen cell suspension was further depleted of T cells by incubation with Dynabeads coated with mouse pan T antibody (Thy 1.2). This T-cell-depleted population of APC was incubated with or without MPL (1 μg/ml) for 24 h, washed with PBS, and fixed with 0.5% paraformaldehyde. The APC (10^6 cells/ml) were then cocultured with the purified CD4$^+$ cells (0.5 × 10^6/ml), suboptimal amounts of anti-CD3 antibody (100 ng/ml), and with or without anti-B7-1 or anti-B7-2 (1 μg/ml) for 5 days (37°C, 5% CO_2). Cultures were pulsed during the last 18 to 20 h of incubation with [^{3}H]TdR, harvested, and the amount of [^{3}H]TdR uptake was determined using a scintillation counter.

Statistics. Statistical analysis (analysis of variance and Student's *t* test) of differences between groups were determined using the InStat computer program (GraphPad Software).

RESULTS

Specific serum responses. We were initially interested in determining the effectiveness of MPL as a mucosal adjuvant in potentiating responses to rHagB. Therefore, groups of mice were immunized by the i.n. route with rHagB plus MPL or rHagB alone and samples of serum and secretion were collected at various times. Serum IgG anti-HagB responses were seen in mice by week 4 following the initial immunization with rHagB and MPL (group B), and they were significantly higher ($P < 0.05$) than those observed in mice immunized with rHagB alone (Fig. 1). The response in group B mice persisted through week 22 with only a slight decrease seen after week 12. A low serum IgG anti-HagB response was seen in mice immunized with antigen alone, and no IgG anti-rHagB antibody activity was detected in serum samples from the nonimmunized mice.

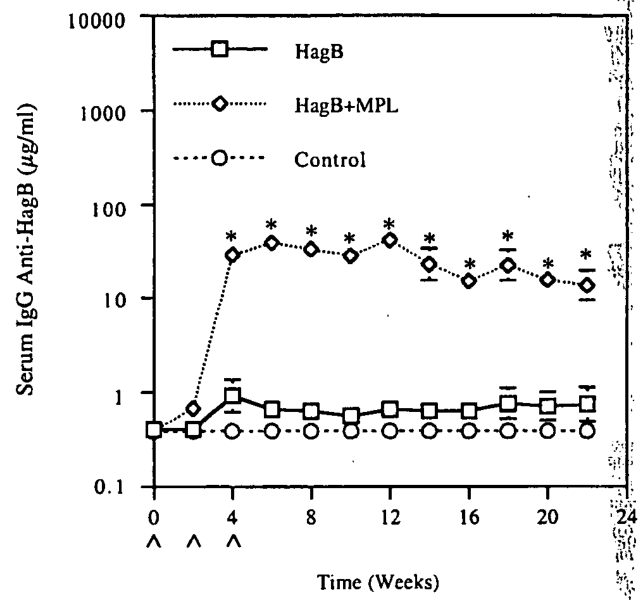
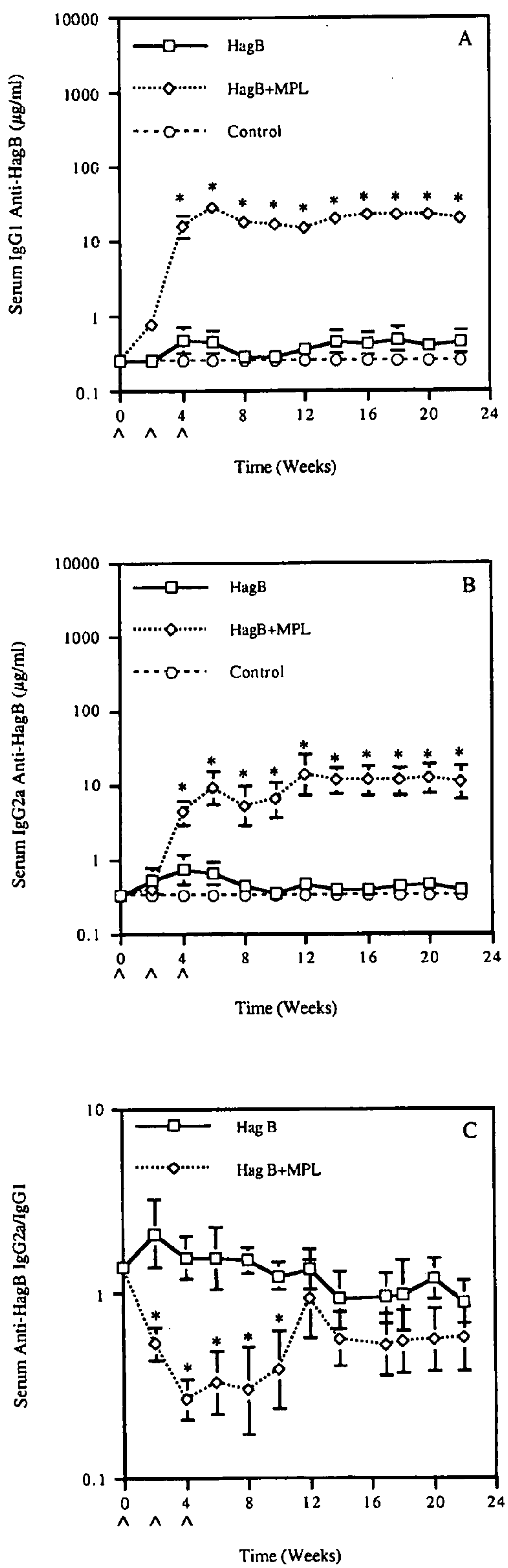


FIG. 1. Time course of the serum IgG anti-rHagB response in mice immunized by the i.n. route with rHagB alone or rHagB plus MPL. Mice were immunized (^) on days 0, 14, and 28 by the i.n. route with rHagB (20 μg) or with rHagB (20 μg) plus MPL (25 μg). Individual serum samples were collected prior to and at approximately 2-week intervals following immunization. Values are expressed as the geometric mean ×/÷ SEM. *, value is significantly different ($P < 0.05$) from those obtained in the HagB-only immunized group.

In order to learn more about the nature of the serum IgG response to HagB and the effect of MPL on the response, we next examined the subclass of the IgG anti-HagB response. In mice immunized with HagB plus MPL (group B), both IgG1 and IgG2a anti-HagB antibody responses were induced which were significantly higher ($P < 0.05$) than those seen in mice immunized with HagB alone (group A) (Fig. 2). The IgG2a/IgG1 ratio of the anti-HagB response in mice immunized with HagB plus MPL was less than 1 and was significantly lower ($P < 0.05$) than the ratio of the responses in mice immunized with HagB alone through week 10 (Fig. 2C). The ratio in the former group increased after week 10 (Fig. 2C), which reflected an increase in the level of IgG2a antibody activity (Fig. 2B). Approximately equal amounts of IgG1 and IgG2a anti-HagB antibodies were detected in mice immunized with HagB alone (Fig. 2). The presence of IgG2a and IgG1 antibodies suggest the participation of Th1 and Th2 cells. It has been shown that Th1 cells effect IgG2a responses, whereas Th2 cells influence the switch towards IgG1 (13). Taken together, our results suggest that MPL potentiated a Th2-like response to HagB. The results further demonstrate the effectiveness of the mucosal route of immunization, i.e., the i.n. route, in inducing serum responses.

Specific salivary responses. In order to determine the effectiveness of rHagB in inducing mucosal IgA responses following i.n. immunization and of MPL in potentiating these responses, individual saliva samples collected throughout the experiment were assessed for the level of IgA antibody activity to rHagB.



Mice immunized with rHagB and MPL (group B) had significantly higher ($P < 0.05$) salivary IgA anti-HagB responses than those seen in mice immunized with rHagB alone (Fig. 3). The response peaked at 4 and 8 weeks following the initial immunization and persisted through week 12. Essentially no salivary IgA antibody activity was seen in mice immunized with rHagB alone. These results indicate that MPL has the ability to augment the salivary response to HagB antigen following i.n. immunization, and they thus provide evidence that MPL is an effective mucosal adjuvant.

Cell stimulation by MPL. In order to determine the mechanism of MPL adjuvanticity, we next investigated the ability of MPL to stimulate murine spleen cell proliferation. Murine spleen cells or purified splenic B cells were incubated with various concentrations of MPL and pulsed with [^{3}H]TdR for the last 18 to 20 h of a 48- or 72-h incubation, respectively. MPL was a potent inducer of cell proliferation (Fig. 4). A stimulation index of 5 was seen in spleen cell cultures incubated with only 10 ng of MPL/ml (Fig. 4A). An approximately fivefold increase was seen with 1 μg of MPL/ml. Maximum stimulation was observed in cultures incubated with 2.5 μg of MPL/ml. Purified B cells were also stimulated by MPL in a dose-dependent manner (Fig. 4B). Maximum stimulation was seen with 10 μg of MPL/ml. The higher sensitivity of spleen cells compared to purified B cells to MPL stimulation is likely due to the various types of APC present in the former cultures. These results provided evidence that MPL, like the parent LPS molecule, is a potent stimulator of cell proliferation.

FACS analysis. The costimulatory molecules B7-1 and B7-2 play an important role in T-cell activation. Therefore, we next assessed by FACS analysis the splenic B-cell and macrophage populations for changes in B7 expression following stimulation with MPL. Analysis of the B220^{+low} and B220^{+high} B-cell subpopulations revealed no or only slight changes in the mean fluorescence intensity of B7-1 and B7-2 expression on the B220^{+low} B cells (Table 1). However, an MPL dose-dependent increase was seen in the expression of B7-2 on the B220^{+high} subpopulation. The mean fluorescence intensity of B7-1 expression on this cell population decreased with increasing amounts of MPL. Since the mean fluorescence intensity of B7-1 on B220^{+low} cells was lower than that of B7-2 with and without MPL stimulation and since an increase in mean fluorescence intensity was only seen in relation to B7-2 expression on the B220^{+high} subpopulation of cells, it appears that the adjuvant MPL preferentially up-regulates B7-2 expression on B cells. The percentage of cells expressing either B7-1 or B7-2 costimulatory molecules increased only in the B220^{+low} B-cell subpopulation after MPL stimulation.

Analysis of the CD11b$^+$ splenic macrophage population revealed an increase in the mean fluorescence intensity of B7-2 and especially B7-1 costimulatory molecules after MPL stim-

FIG. 2. Time course of the serum anti-HagB IgG1 (A) and IgG2a (B) responses and the IgG2a/IgG1 ratios (C) in mice immunized by the i.n. route with rHagB or rHagB plus MPL. Values for serum IgG1 and IgG2a antibody activities are expressed as the geometric mean ×/÷ SEM. *, value is significantly different ($P < 0.05$) from those obtained in the HagB-only immunized group.

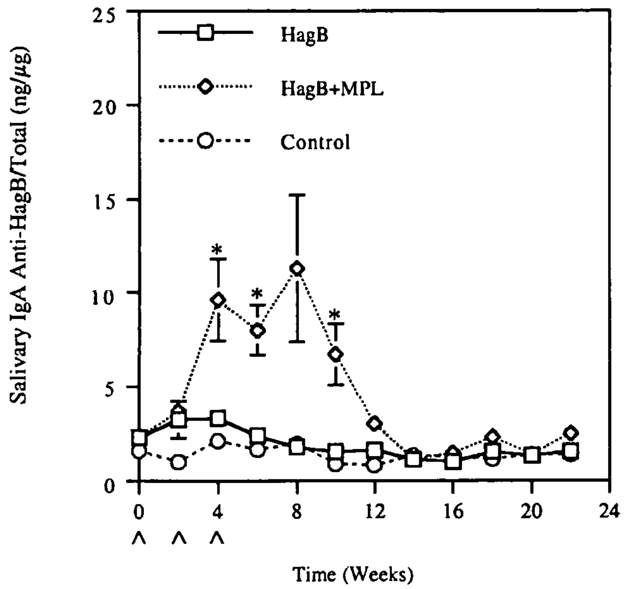
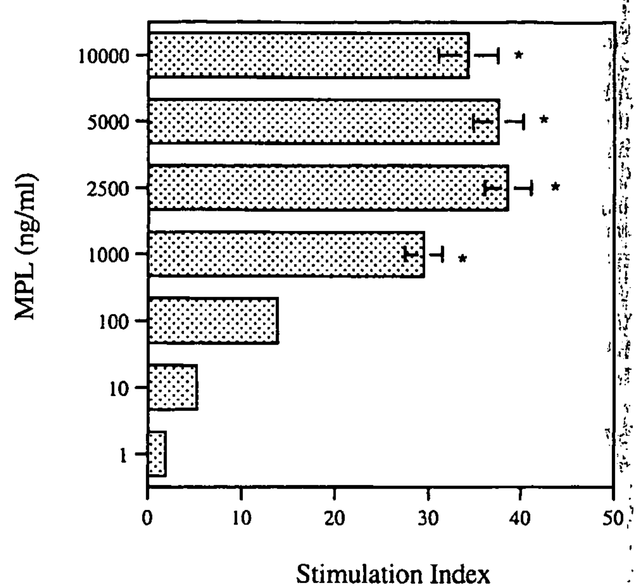


FIG. 3. Time course of the salivary IgA anti-rHagB antibody response in mice immunized by the i.n. route with rHagB alone or rHagB plus MPL. Values are the geometric mean ×/÷ SEM. *, value is significantly different ($P < 0.05$) from those obtained in the HagB-only immunized group.



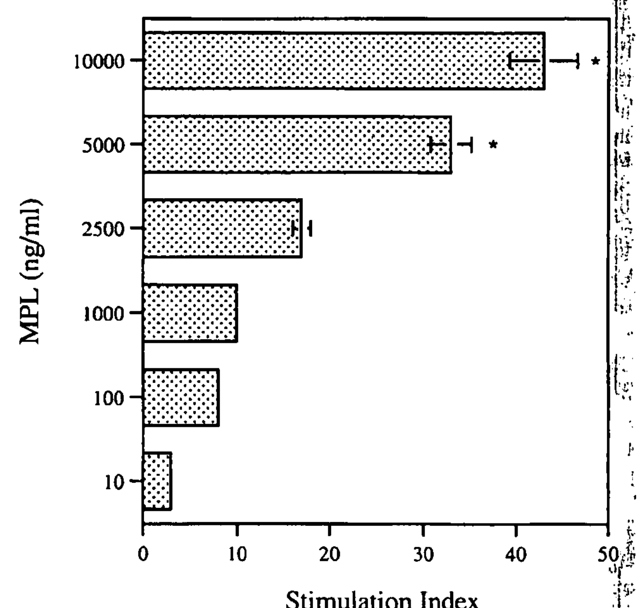


FIG. 4. The effect of MPL on proliferative responses of murine spleen cells and purified B cells. Spleen cells (A) or purified splenic B cells (B) were cultured with various concentrations of MPL for 48 or 72 h, respectively. Cultures were pulsed with [^{3}H]TdR during the last 18 to 20 h. Values are expressed as the stimulation index. The mean background activities in the spleen cell and purified B-cell cultures were 1,424 ± 320 cpm and 497 ± 77 cpm, respectively. Results are representative of three separate experiments. *, value is significantly different ($P < 0.001$) from those obtained with spleen cell cultures stimulated with 1 ng of MPL/ml or purified splenic B cells stimulated with 10 ng of MPL/ml.

ulation (Table 2). Furthermore, analysis of the CD11c$^+$ dendritic cell population following stimulation with MPL revealed an increase in the mean fluorescence intensity of B7-1 (683) and B7-2 (1,486) expression above that seen in unstimulated cultures (B7-1, 397; B7-2, 726). These results demonstrate that MPL can stimulate B7-1 and B7-2 on macrophages, as has been shown for LPS (18, 28). Taken together, our results suggest a differential regulation of B7-1 and B7-2 on B cells and on macrophages and dendritic cells by MPL. That is, MPL induced an increase in B7-2 but not B7-1 expression on B cells (B220$^{+\text{high}}$), whereas an increase was especially seen in B7-1 expression on macrophages.

Assessment of cocultured cells. Although FACS analysis indicated that MPL stimulation affects the expression of the costimulatory molecules B7-1 and B7-2, we wanted to further assess the importance of this effect of MPL in a functional assay using cocultures of APC and T cells. The addition of anti-B7-1 or -B7-2 antibody to cultures of APC and CD4 T cells resulted in a 73 or 81% reduction in proliferative responses, respectively, compared to control cultures (Fig. 5). These results indicate the involvement of B7-1 and especially B7-2 in the immunoadjuvant effect of MPL in this system.

DISCUSSION

With the advent of more sophisticated and refined techniques, the current trend is to use purified recombinant, subunit, or synthetic antigens for mucosal vaccine development. However, these antigens when given alone are generally poorly immunogenic. Therefore, the success of a future mucosal vaccine will be contingent upon the use of an appropriate adjuvant. MPL is a promising adjuvant for vaccine development. MPL is a nontoxic derivative of LPS which has retained the ability to enhance immune responses (58). In the present study, we investigated the immunoadjuvant properties of MPL in conjunction with the *P. gingivalis* hemagglutinin antigen

TABLE 1. Effect of MPL on B7-1 and B7-2 expression on murine splenic B cells[a]

MPL (ng/ml)	Mean fluorescence intensity			
	B220+low		B220+high	
	B7-1	B7-2	B7-1	B7-2
0	15	48	43	61
10	16 (7)[b]	48 (7)	32 (<1)	89 (<1)
100	18 (9)	56 (9)	35 (<1)	100 (<1)
1,000	16 (13)	54 (12)	23 (<1)	115 (<1)
10,000	16 (10)	58 (11)	23 (<1)	105 (<1)

[a] Single spleen cell cultures were incubated in the presence or absence of MPL for 48 h. Cells were harvested and stained with allophycocyanin-conjugated anti-B220 and FITC-labeled anti-CD86 or PE-labeled anti-CD80 antibody and analyzed by flow cytometry.

[b] Values in parentheses are the increase in the percentage of B7-1- and B7-2-expressing cells in the B220+low and B220+high populations.

HagB as a potential mucosal vaccine against periodontal disease. Furthermore, we have investigated the role of the costimulatory molecules B7-1 and B7-2 on APC in the adjuvant activity of MPL. We have demonstrated that i.n. immunization of mice with rHagB and MPL resulted in a significantly higher serum IgG anti-HagB response than that seen in mice immunized with antigen alone. Furthermore, the level of specific salivary IgA antibody observed was also significantly higher when rHagB was given with MPL. These data demonstrate that MPL is an effective adjuvant when coadministered with the potential virulence antigen HagB. To our knowledge this is the first study in which MPL has been used in combination with an antigen derived from a periodontal pathogen.

Previous studies have provided evidence suggesting that MPL can act as a mucosal adjuvant with various potential vaccine antigens (8, 42, 53). In one study (42), we reported that i.n. immunization of mice with MPL and the recombinant saliva-binding region (SBR) of *Streptococcus mutans* AgI/II adhesin induced higher serum and mucosal antibodies than the response seen in mice immunized with SBR alone. Moreover, the responses peaked 2 weeks after the last (third) immunization and were maintained for about 17 weeks. Our present findings indicate that both serum IgG and salivary IgA responses reached peak levels 2 weeks after the second immunization, and while the serum response was maintained at a high level until the termination of the experiment (≈22 weeks), salivary IgA antibody activity decreased to background levels

TABLE 2. Effect of MPL on B7-1 and B7-2 expression on murine splenic macrophages[a]

MPL (ng/ml)	Mean fluorescence intensity[b]	
	B7-1	B7-2
0	575 ± 49	259 ± 47
100	1,844 ± 196**	358 ± 24**
1,000	1,644 ± 144**	339 ± 24
10,000	779 ± 123	342 ± 21*

[a] Single spleen cell cultures were incubated in the presence or absence of MPL for 48 h. Cells were harvested and stained with allophycocyanin-conjugated anti-CD11b and FITC-labeled anti-CD86 or PE-labeled anti-CD80 antibody and analyzed by flow cytometry. Values are the mean ± SEM.

[b] *, significantly different from control (no MPL) at $P < 0.01$; **, significantly different from control (no MPL) at $P < 0.001$.

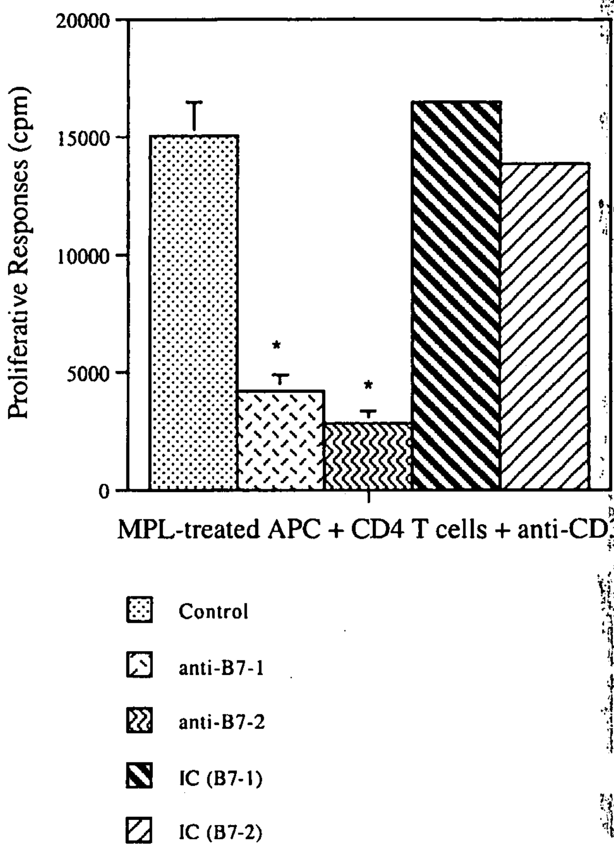


FIG. 5. The involvement of B7-1 and B7-2 in MPL-induced proliferative activity of purified CD4+ T cells. Splenic APC were incubated with MPL (1 μg/ml) for 24 h and then cocultured with purified CD4+ cells and anti-CD3, with or without anti-B7-1 or anti-B7-2 or isotype control for 5 days. Cultures were pulsed with [3H]TdR for the last 18 to 20 h of incubation. Values are expressed as the mean counts per minute ± SEM. Results are representative of three separate experiments. *, value is significantly different ($P < 0.001$) from those obtained with control cultures.

by week 14. The regimen of immunization used in our studies was based on those reported by Wu and Russell (62), who showed an increase in both serum IgG and salivary IgA antibody activities after a third i.n. immunization with AgI/II. It is interesting that following i.n. immunization of rats with MPL and rHagB, using the same basic protocol, a serum IgG response but not a notable salivary IgA response was induced (unpublished observation). Therefore, it is likely that the differences seen in the salivary IgA responses in other studies (8, 42, 53) compared to the present one are due to differences in the nature of the antigens.

With regards to the subclass of the serum IgG antibody responses induced following immunization, the presence of IgG2a and IgG1 antibodies are indicative of a mixed type 1 and type 2 immune response (15, 44). A number of studies have reported a shift to a type 1 response following immunization with MPL as adjuvant (2, 43, 46, 53). Other studies by De-Becker et al. (10) demonstrated in vivo and in vitro that MPL drives the development of cells that secrete type 1 and type 2 cytokines and the induction of IgG2a and IgG1 antibodies,

respectively. In our study, we observed the induction of a type 1 and type 2 response following immunization with HagB, as suggested by the subclass of the IgG anti-HagB response, whereas immunization with HagB plus MPL resulted in an increase in the level of serum IgG1 antibody activity. These findings suggest that the nature of the response was mainly influenced by the HagB antigen and that MPL amplified a type 2 response. One possibility that a type 2 response is favored may relate to the ability of MPL to up-regulate B7 expression on APC. Studies have reported that B7-1 and B7-2 have differential effects on T-cell differentiation (31, 35, 57), whereas other evidence does not support these findings (7, 33). Thus, the increase in the type 2 response observed in the present study may not be related to the differential increase in the B7 molecules on different APC. Interestingly, Broeren et al. (4) demonstrated that Th cell activation by either B7-1 or B7-2 leads to the production of Th2-like cytokines, e.g., IL-4, IL-10, and IL-13. Another possibility for the augmented type 2 response is the ability of MPL to enhance the production of IL-10, which contributes to a decreased production of IL-12 and IFN-γ mRNA (52). Finally, another possibility could involve the interplay between MPL and specific Toll-like receptors. Current studies in our laboratory are investigating these possibilities.

Previous studies in rats have also reported the induction of a mixed type 1 and type 2 serum IgG anti-HagB response following subcutaneous immunization with rHagB in complete Freund's adjuvant (27). This was somewhat surprising, since complete Freund's adjuvant favors the induction of type 1 responses to coadministered antigens and, thus, further implicates the role of HagB in the nature of the response. Studies by Kohler et al. (30) have shown that oral administration of a *Salmonella* strain expressing the cloned HagB to mice results in the induction of a predominant systemic IgG2a anti-HagB response. These results were similar to those obtained by others who used a *Salmonella* vector vaccine system (49, 59). Since *Salmonella* induces a type 1 response, these investigators suggested that the vector influenced the response to the expressed cloned antigen. However, our laboratory reported a mixed type 1 and type 2 response to a cloned antigen of *S. mutans* expressed by a *Salmonella* strain (17). These finding suggests that the property of the cloned antigen influenced the response and that the nature of the response was not entirely determined by the *Salmonella* vector.

It is well known that at least two signals are necessary for an immune response, i.e., antigen presentation to T cells in the context of peptide-MHC class II molecule(s) and the interaction of costimulatory molecules on APC with T cells. Based on our results and those of other investigators (10), it appears that the immunoadjuvanticity of MPL is associated with its potential to activate APC. We have shown in the present study that MPL stimulates proliferation of purified murine B cells and, as previously shown (21), of whole spleen cell cultures. Furthermore, FACS analysis showed a preferential up-regulation of B7-2 expression on the B220$^{+\text{high}}$ subpopulation of B cells. Lenschow et al. (34) demonstrated that cross-linking of surface immunoglobulin on B220^{+} cells induced B7-2 but not B7-1 expression and that these cells were able to stimulate T-cell proliferation. However, these investigators did not separate the B220^{+} cells into high- and low-intensity subpopulations. In the present study, we also observed an increase in the percentage of cells expressing either B7-1 or B7-2 only in the B220$^{+\text{low}}$ subpopulation of cells. Interestingly, the B220$^{+\text{low}}$ cells have been shown to be the subpopulation of B cells involved in immunoglobulin secretion and proliferation (45).

Converse to the preferential up-regulation of B7-2 on B cells, we observed an up-regulation of B7-2 and especially of B7-1 on macrophages following incubation with MPL. Studies by DeBecker et al. (10) have reported that the expression of B7-2 on macrophages remained unchanged after MPL stimulation. However, these investigators did not observe an up-regulation of B7-2 on B cells. This difference in results may reflect the culture conditions used, since the present study shows differences in the expression of B7-1 and B7-2 not only between B-cell subpopulations but also between B cells and macrophages. Thus, the ability of both the B7-1 and B7-2 antibodies to inhibit the proliferative activity of purified CD4^{+} T cells (Fig. 5) can be explained by the various types of APC present in the cultures, including CD11c^{+} dendritic cells, which exhibited an increase in the mean fluorescence intensity of B7-1 and B7-2 following MPL stimulation.

In the present study we have shown that MPL is an effective adjuvant in promoting mucosal and serum antibody responses when given with rHagB from the periodontal pathogen *P. gingivalis* by the i.n. route. Furthermore, we have shown that MPL differentially modulates the expression of the B7 costimulatory molecules on subpopulations of B cells and macrophages. Current studies are further investigating the mechanisms of MPL adjuvanticity in order to better design vaccines which will potentiate optimal protective responses against infectious agents.

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